ELECTRONIC SUPPLEMENTARY INFORMATION

A minimal structural variation can overcome tumour resistance of oxaliplatin: the case of 4,5-dehydrogenation of the cyclohexane ring.

Paride Papadia,a,b,* Valentina Gandin,c* Alessandra Barbanente,b,d Alessandro G. Ruello,b,d Cristina Marzano,c Katia Micoli,b,d James D. Hoeschele,e Giovanni Natile,b,d Nicola Margiotta.b,d,*

aDepartment of Biological and Environmental Sciences and Technologies (DiSTeBA), University of Salento, 73100 Lecce, Italy;
bCIRCMSB, Consorzio Interuniversitario di Ricerca in Chimica dei Metalli nei Sistemi Biologici, 70121 Bari, Italy;
cDipartimento di Scienze del Farmaco, Università di Padova, Via Marzolo 5, 35131, Padova, Italy.
dDipartimento di Chimica, Università degli Studi di Bari Aldo Moro, Via E. Orabona 4, 70125 Bari, Italy;
eDepartment of Chemistry, Eastern Michigan University, 48197 Ypsilanti, MI (USA).

CONTENT:

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Biological investigation

NMR characterization of the compounds
Experimental section

Materials and methods

Commercial reagent grade chemicals and solvents were used as received without further purification. $^1$H-NMR, $^{13}$C-NMR, and NOESY 2D NMR spectra were recorded on a Bruker Avance III 700 MHz instrument. $^1$H and $^{13}$C chemical shifts were referenced using internal residual peak of the solvent (DMSO-$d_6$: 2.50 ppm for $^1$H and 39.51 ppm for $^{13}$C; D$_2$O: 4.8 ppm for $^1$H; CD$_3$OD: 3.31 ppm for $^1$H and 49 ppm for $^{13}$C; N,N-dimethylformamide-$d_7$: 8.03 ppm for $^1$H).

Electrospray ionization mass spectrometry (ESI-MS) was performed with electrospray interface and an ion trap mass spectrometer (1100 series LC/MSD Trap system Agilent, Palo Alto, CA). Elemental analyses were carried out with an Eurovector EA 3000 CHN instrument.

Synthesis and characterization of Platinum complexes

$K_2[PtCl_4]$, $cis$-[Pt(CBDCA)(DMSO)$_2$]$^1$ (CBDCA = 1,1-ciclobutane dicarboxylate), and $cis$-[PtCl$_2$(DMSO)$_2$]$^2$ (DMSO = dimethyl sulfoxide), were prepared according to already reported procedures. The elemental analysis and spectroscopic data of the precursor compounds were analogous to those reported in the literature.

Synthesis of $cis$-[PtCl$_2$(DACHEX)] (1). $K_2[PtCl_4]$ (100 mg, 0.241 mmol) was dissolved in 10 mL of water and treated with 10 mL of a solution containing trans-1,2-diamine-4-cyclohexene dihydrochloride (44.6 mg, 0.241 mmol) neutralized with KOH (27.0 mg, 0.48 mmol). The mixture was stirred at room temperature and after a few minutes a yellow precipitate started to form. After 3 h incubation, the pH of the suspension was brought to 8.0 by addition of KOH (1M) and the suspension was stirred at room temperature for further 17 h. The yellow precipitate was isolated by filtration of the mother liquor, washed with a small amount of water and dried under vacuum. Yield 78.5 % (72 mg, 0.190 mmol). Anal.: calculated for $C_6H_{12}Cl_2N_2Pt\cdot H_2O$ ($1\cdot H_2O$) C, 18.19; H, 3.56; N, 7.07 %. Found: C, 18.38; H, 3.24; N, 7.11 %. ESI-MS$^+$ (DMSO solution): calculated for $C_8H_{18}ClN_2OPt\cdot DMSO$+: 420.0476. Found: m/z 420.0453. $^1$H-NMR (DMSO-$d_6$): 5.75 (2H, NHa), 5.34 (2H, CHf), 5.17 (2H, NHb), 2.72 (2H, CHc), 2.31 (2H, Chc), 2.14 (2H, Chd) ppm. $^1$H-NMR (N,N-Dimethylformamide-$d_7$): 5.82 (2H, NHa), 5.49 (2H, CHf), 5.20 (2H, NHb), 2.54 (2H, Chc), 2.40 (2H, Chd) ppm.

Synthesis of $cis$-[PtI$_2$(DACHEX)] (2). $K_2[PtCl_4]$ (100 mg, 0.241 mmol) was dissolved in 5 mL of water and treated with 320 mg of KI (8-fold excess). The reaction mixture was stirred at room temperature for 20 min and then treated with 1.5 mL of a solution containing trans-1,2-diamine-4-cyclohexene dihydrochloride (44.6 mg, 0.241 mmol) neutralized with KOH (27 mg, 0.482 mmol). A precipitate formed immediately, and the resulting suspension was stirred at room temperature for 3 h. The brown precipitate was isolated by filtration of the mother liquor, washed with a small amount of water (1 mL) and dried under vacuum. Yield 77.2% (117 mg, 0.209 mmol). Anal.: 1. R. Ranaldo, N. Margiotta, F.P. Intini, C. Pacifico, G. Natile. Inorg. Chem. 2008, 47, 1820. 2. Y.N. Kukushkin, Y.E. Vyaz’menkii, L.I. Zorina, Y.L. Pazukhina. Zh. Neorg. Khim. 1968, 13, 1595.
calculated for C₆H₁₂N₂I₂Pt·1.5H₂O (2·1.5H₂O): C, 12.25; H, 2.57; N, 4.76 %. Found: C, 12.16; H, 2.09; N, 4.64 %. ESI-MS⁺ (DMSO solution): calculated for C₈H₁₈N₂OPTS [2⁻I⁺DMSO]⁺: 511.9832. Found: m/z 511.9815. ¹H-NMR (D₂O): 5.49 (2H, CHf) 2.74 (2H, CHc) 2.58 (2H, CHe) 2.18 (2H, CHd) ppm. ¹H-NMR (N,N-Dimethylformamide-d₇): 5.75 (2H, NHa), 5.46 (2H, CHf), 5.06 (2H, NHb), 2.78 (2H, CHc), 2.63 (2H, CHe), 2.42 (2H, CHd) ppm.

**Synthesis of [Pt(CBDCA)(DACHEX)] (3).** To a solution of [Pt(CBDCA)(DMSO)²] (200 mg, 0.405 mmol) in 125 mL of water was added, dropwise, 1.5 mL of a solution containing a trans-1,2-diamine-4-cyclohexene dihydrochloride (75 mg, 0.405 mmol) previously neutralized with KOH (45 mg, 0.81 mmol). The reaction mixture was refluxed for 2 h. The resulting mixture was refluxed for 2 h. The resulting suspension was concentrated under reduced pressure to a minimum volume and the light-yellow solid was separated by filtration of the mother liquor, washed with 0.5 mL of cold water, and dried under vacuum. Yield 49.6% (90 mg, 0.201 mmol). Anal.: calculated for C₁₂H₁₈N₂O₄Pt·H₂O (3·H₂O): C, 30.83; H, 4.31; N, 5.99 %. Found: C, 30.30; H, 4.25; N, 6.05 %. ESI-MS⁺: calculated for C₁₂H₁₈N₂O₄PtNa [3⁺Na]⁺: 472.0812. Found: m/z 472.0879. ¹H-NMR (CD₃OD): 5.47 (2H, CHf), 2.88 (4H, CHg), 2.61 (2H, CHc), 2.53 (2H, CHe), 2.19 (2H, CHd), 1.86 (2H, CHh) ppm. ¹³C (CD₃OD): 14.82, 30.43, 32.02, 56.10, 59.01, 124.11, 180.47 ppm.

**Synthesis of [Pt(OXA)(DMSO)₂] (OXA = oxalate).** A solution containing cis-[PtCl₂(DMSO)₂] (250 mg, 0.59 mmol) in 125 mL of water was treated with AgNO₃ (196 mg, 1.15 mmol). The reaction mixture was stirred in the dark at 50 °C for 6 h and then at room temperature overnight. The resulting suspension was filtered through celite and the filtrate treated with 1.5 mL of a solution containing oxalic acid (74.6 mg, 0.59 mmol) neutralized with KOH (66.4 mg, 1.18 mmol). The resulting mixture was stirred at room temperature for 24 h meanwhile some precipitate formed. The solvent was removed under reduced pressure and the white residue was washed with a small amount of cold water (1 mL) and dried under vacuum. Yield 79.04 % (205 mg, 0.467 mmol). ¹H-NMR (D₂O): 3.66 ppm (12H).

**Synthesis of [Pt(OXA)(DACHEX)] (4).** [Pt(DMSO)²(OXA)] (200 mg, 0.46 mmol) was dissolved in 280 mL of water and treated with a solution containing trans-1,2-diamine-4-cyclohexene dihydrochloride (73 mg, 0.39 mmol) neutralized with KOH (44 mg, 0.79 mmol) added dropwise. The reaction mixture was refluxed for 2 h and the resulting suspension was filtered and the solution concentrated under reduced pressure to a minimum volume (5 mL). The light-yellow solid that precipitated from the mother liquor was isolated by filtration, washed with a small amount of cold water (1 mL), and dried under vacuum. Yield 63 % (114 mg, 0.29 mmol). Anal.: calculated for C₈H₁₂N₂O₄Pt·H₂O (4·H₂O): C, 23.24; H, 3.41; N, 6.77 %. Found: C, 23.41; H, 3.00; N, 6.88 %. ESI-MS⁺: calculated for C₈H₁₂N₂O₄PTa[4⁺Na]⁺: 418.0342. Found: m/z 418.0329. ¹H-NMR (D₂O): 5.49 (2H, CHf), 2.74 (2H, CHc), 2.61 (2H, CHe), 2.22 (2H, CHd) ppm. ¹H-NMR (DMSO-d₆): 6.27 (2H, NHa) 5.49 (2H, NHb) 5.38 (2H, CHf), 2.34 (2H, CHc), 2.32 (2H, CHe), 2.12 (2H, Chd). ¹³C-NMR (D₂O): 32.79, 59.42, 125.13, 168.96.
Cytotoxicity assays

Complex solubilisation

Complexes 1 and 2 were dissolved immediately before the experiments in dimethyl sulfoxide, and calculated amounts of drug solution were added to the cell growth medium, to a final DMSO concentration of 0.5%, having no discernible effect on cell vitality. Cisplatin, oxaliplatin, 3 and 4 were dissolved in 0.9% aqueous NaCl.

Cellular cultures

Human colon (LoVo and HCT-15) carcinoma cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). 2008 human ovarian cancer cells were kindly provided by Prof. G. Marverti (Dept. of Biomedical Science of Modena University, Italy). Human cervical carcinoma (A431) cells were kindly provided by Prof. F. Zunino (Division of Experimental Oncology B, Istituto Nazionale dei Tumori, Milan, Italy). The multidrug-resistant sub-line (LoVo MDR) was kindly provided by Prof. F. Majone (Dept. of Biology of Padova University, Italy). The LoVo-OXP cells were derived, using a standard protocol, by growing LoVo cells in increasing concentrations of oxaliplatin and following nine months of selection of resistant clones. Cell lines were maintained in the logarithmic phase at 37 °C in a 5% carbon dioxide atmosphere using the following culture media containing 10% fetal calf serum (Euroclone, Milan, Italy), antibiotics (50 units per mL penicillin and 50 μg mL⁻¹ streptomycin), and 2 mM L-glutamine: (i) RPMI-1640 medium (Euroclone) for 2008, HCT-15, and A431 cells; (ii) F-12 HAM’S (Sigma Chemical Co.) for LoVo, LoVo MDR, and LoVo-OXP cells.

MTT assay

The growth inhibitory effect toward tumor cell lines was evaluated by MTT test. Briefly, 3·8·10³ cells/well, dependent upon the growth characteristics of the cell line, were seeded in 96-well microplates in growth medium (100 µL) and then incubated at 37 °C in a 5% carbon dioxide atmosphere. After 24 h, the medium was removed and replaced with a fresh one containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After 72 h, each well was treated with 10 µL of a 5 mg·mL⁻¹ MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) saline solution, and after 5 h of incubation, 100 µL of a sodium dodecylsulfate (SDS) solution in HCl 0.01 M were added. Following an overnight incubation, the inhibition of cell growth induced by the tested complexes was evaluated by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 microplate reader.

Cellular uptake

LoVo OXP cells (3·10⁶) were seeded in 75 cm² plates with 20 mL growth medium. After overnight incubation, the medium was replaced and the cells were treated with the tested compounds for
24 h. Cell monolayers were washed twice with cold PBS, harvested and counted. Samples were then subjected to three freezing/thawing cycles at -80 °C, and then vigorously vortexed. The samples were treated with highly pure nitric acid (Pt: \(<0.01 \mu g\cdot kg^{-1}\), TraceSELECT® Ultra, Sigma Chemical Co.) and transferred into a microwave teflon vessel. Subsequently, samples were submitted to standard procedures using a speed wave MWS-3 Berghof instrument (Eningen, Germany). After cooling, each mineralized sample was analyzed for platinum by using a Varian AA Duo graphite furnace atomic absorption spectrometer (Varian, Palo Alto, CA; USA) at the wavelength of 324.7 nm. The calibration curve was obtained using known concentrations of standard solutions purchased from Sigma Chemical Co..

**DNA platination.**

LoVo OXP cells (5·10⁶) were seeded in 10 cm Petri dishes containing 10 mL of culture medium. Subsequently, cells were treated with the tested complexes for 24 h. DNA was extracted and purified by a commercial spin column quantification kit (Qiagen DNeasy Blood and Tissue Kit). Only highly purified samples (A260/A230 = 1.8 and A280/A260 = 2.0) were included for analysis to avoid any artefacts. The samples were completely dried and re-dissolved in 200 μL of Milli-Q water (18.2 MΩ) kept for at least 20 min at 65 °C in a shaking thermo-mixer, mineralized, and analysed for total Pt content by GF-AAS as described above.

**Transmission electron microscopy analyses**

About 106 LoVo OXP cells were seeded in 24-well plates and, after 24 h incubation, treated with the tested compounds and incubated for additional 36 h. Cells were then washed with cold PBS, harvested and directly fixed with 1.5% glutaraldehyde buffer with 0.2 M sodium cacodylate, pH 7.4. After washing with the buffer and post-fixation with 1% OsO4 in 0.2 M cacodylate buffer, specimens were dehydrated and embedded in epoxy resin (Epon Araldite). Sagittal serial sections (1 μm) were counterstained with toluidine blue; thin sections (90 nm) were given contrast by staining with uranyl acetate and lead citrate. Micrographs were taken with a Hitachi H-600 electron microscope (Hitachi, Tokyo, Japan) operating at 75 kV. All photos were typeset in Corel Draw 11.
Scheme 1. Synthesis of complex 1.

Figure S1. $^1$H-NMR (700 MHz, $^1$H) spectrum of 1 in DMF-d$_7$. The asterisks mark residual solvent peaks. Protons Hc are obscured by the solvent signal.
Figure S2. $^1$H-NMR (700 MHz, $^1$H) spectrum of 2 in D$_2$O. The asterisk marks a residual solvent peak.
Figure S3. $^1$H-NMR (700 MHz, $^1$H) spectrum of 2 in DMF-d$_7$. The asterisks mark residual solvent peaks.
Figure S4. $^1$H- NMR (700 MHz, $^1$H) spectrum of compound 3 in CD$_3$OD. The asterisks indicate residual solvent peaks.
Scheme 2. Synthesis of cis-[Pt(OXA)(DMSO)$_2$].

Scheme 3. Synthesis of compound 4.
Figure S5. $^1$H-NMR (700 MHz, $^1$H) spectrum of compound 4 in DMSO-d$_6$. The asterisks indicate residual solvent peaks.

Figure S6. $^1$H-NMR (700 MHz, $^1$H) spectrum of compound 4 in D$_2$O. The asterisk indicates residual solvent peak.