Supplementary material

NMR spectroscopy: ^{195}Pt NMR and ^{1}H NMR data were recorded on Varian Unity-500 MHz and Varian Unity-300 MHz spectrometers respectively and data were processed using MestreNova software.

The purities of tested compounds are ≥95% (HPLC). HPLC analyses were performed on a Varian ProStar HPLC system equipped with a UV detector, set at 220 nm, using a RP-C18 column (Phenomenex, Luna, 250 x 4.6 mm, 5µm, 100Å).

Synthesis of compounds

Compounds I-IV were prepared as previously reported (ref 25-26)

trans- [$PtCl_2$(n-butylamine) (VPA)(OH) (pip-pip)] (V)

trans-[$PtCl_2$(*n*-butylamine)(pip-pip)]$^+$ was oxidized with H_2O_2 as described above to yield *trans*-[$PtCl_2$(*n*-butylamine)$(OH)_2$(pip-pip)]$^+$. To a solution of *trans*-[$PtCl_2$(*n*-butylamine)$(OH)_2$(pip-pip)]$^+$ (21 mg, 3.65×10^{-5} mol) in DMF (1 mL), valproic anhydride (22 µL, 7.3×10^{-5} mol) was added and stirred overnight at rt. The solvent was evaporated and diethyl ether was added to precipitate the desired compound as a light yellow powder.

Yield: 14 mg, 55% ^{195}Pt-NMR (D_2O): 867 ppm. ^{1}H-NMR (D_2O): δ ppm 0.74 (t, J = 7.32 Hz, 6H), 0.79 (t, J = 7.32 Hz, 3H), 1.17 (m, 4H), 1.27 (m, 7H), 1.62 (m, 5H), 1.88 (m, 4H), 2.14 (m, 2H), 2.27 (m, 1H), 2.73 (t, J = 7.47 Hz, 2H), 2.90 (t, J = 12.1 Hz, 2H), 3.01 (t, J = 12.9 Hz, 2H), 3.39 (m, 5H). Elemental analysis % calcd. for $C_{22}H_{48}Cl_3N_3O_3Pt$: C, 37.53; H, 6.87; N, 5.97. Found: C, 37.25; H, 7.11; N, 6.05.

ctc-[$Pt(NH_3)_2(PhB)_2Cl_2$] (VI)

ctc-[$Pt(NH_3)_2(OH)_2Cl_2$] (100 mg, 2.99×10^{-4} mol) was suspended in N,N-dimethylformamide (1 mL) and 4-phenylbutyric anhydride was added (232 mg, 7.47×10^{-4} mol, 2.5 eq). The reaction mixture was stirred for 1h (complete dissolution) at 40°C. The solvent was evaporated at reduced pressure. The residue was re-dissolved in acetone and diethylether was added to precipitate the compound. The precipitate was collected by filtration, washed twice with diethyl ether and dried in vacuo.

Yield: 138 mg, 74% ^{195}Pt-NMR (DMF):1174 ppm. ^{1}H-NMR (DMSO): δ ppm 1.72 (m, 4H), 2.20 (t, 4H), 2.57 (t, 4H), 6.27-6.80(b, 6H), 7.24(m, 10H). Elemental analysis % calcd. for

$C_{20}H_{28}Cl_2N_2O_4Pt$: C 38.35; H 4.51; N, 4.47; found: C 38.90, H 4.75, N 4.59

ctc-[$Pt(NH_3)_2(OAc)(PhB)Cl_2$] (VII)

To a solution of ctc-[$Pt(NH_3)_2(OAc)(OH)Cl_2$] (50 mg,1.32×10^{-4}mol) in N,N-dimethylformamide (2mL), 4-phenylbutyric anhydride (62 mg, 1.99×10^{-4}mol, 1.5 eq) was added. After stirring for 1h at rt, solvent was evaporated at reduced pressure. Acetone was added to the residue and the precipitated compound was collected by filtration, washed twice with acetone, and dried in vacuo to afford the desired compound as a light yellow powder.

Yield: 60 mg, 87% ^{195}Pt-NMR(DMF):1176PPM, ^{1}H-NMR (DMSO): δ ppm 1.704 (m, 2H), 1.87(s, 3H), 2.18(t,2H), 2.55(t,2H), 6.5 (b,6H), 7.18(m, 5H). Elemental analysis % calcd. for

$C_{12}H_{20}Cl_2N_2O_4Pt$: C, 27.60; H 3.86; N, 5.36; found: C 28.07, H 4.08, N 3.93

[Pt(DACH)(PhB)$_2$(ox)] (VIII)

To a suspension of [Pt(DACH)(OH)$_2$(ox)] (100 mg, 2.32 x 10^{-4} mol) in N,N-dimethylformamide (1 mL), 4-phenylbutyric anhydride (180 mg, 5.8x10^{-4} mol, 2.5 eq) was added. The mixture was stirred at RT for 2.5 h. Solvent was evaporated at the end of the reaction and dichloromethane was added to precipitate the desired compound as a white powder. The compound was separated by centrifugation, washed twice with dichloromethane and dried under vacuo.

Yield: 121 mg, 72% ^{195}Pt-NMR(DMF): 1588 ppm, . ^{1}H-NMR (DMSO): δ ppm 1.0-1.59(m, 8H), 1.69 (m, 4H), 2.02-2.34 (m, 8H), 7.2(m, 10H), 8.32(b, 4H). Elemental analysis % calcd. for $C_{28}H_{36}N_2O_8Pt$: C 46.47; H 5.01; N, 3.87; found: C 46. 8, H 5.23, N 3.64

Measurement of the partition coefficient

To determine the partition coefficient (*P*) the shake flask method was used. Octanol-saturated water (OSW) and water-saturated octanol (WSO) were prepared using analytical grade octanol and 0.2 M aqueous NaCl solution (to suppress hydrolysis of the chlorido complexes). Compounds were first dissolved in DMF. Mixing was done by vortexing for 30 min at room temperature to establish the partition equilibrium. To separate the phases, centrifugation was done at 3000 x g for 5 min. The aqueous layer was carefully separated from the octanol layer for platinum analysis. Pt was quantified from aliquots taken from the octanol-saturated aqueous samples before and after partition by FAAS. Partition coefficients of Pt complexes were calculated using the equation log *P* = log ([Pt]WSO/[Pt]OSW).

Experiments with Cultured Human Cells. Platinum(IV) complexes were dissolved in DMSO to stock solutions of 1 mg/mL just before the experiment, and a calculated amount of drug solution was added to the cell growth medium to a final solvent concentration of 0.5%, which had no discernible effect on cell killing. Cisplatin (CDDP) and oxaliplatin (OXP) were dissolved just before the experiment in a 0.9% NaCl solution.

Cisplatin, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 4-phenylbutyric acid, staurosporin, Z-VAD-fmk and Hoechst 33258 (2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride hydrate) were obtained from Sigma Chemical Co, St.Louis, USA.

Cell cultures. Human lung (A549), breast (MCF-7), pancreatic (BxPC3), kidney (A498) and colon (HCT-15) carcinoma cell lines along with melanoma (A375) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Human prostate adenocarcinoma (PC3) cells were obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK). Human ovarian cancer cell lines A2780 and its cisplatin resistant variant, A2780cisR, were kindly provided by Prof. G. Marverti (Dept. of Biomedical Science of Modena University, Italy. 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·mL^{-1} penicillin and 50 µg·mL^{-}1 streptomycin) and 2 mM L-glutamine: i) RPMI-1640 medium (Euroclone) for MCF-7, HCT-15, A431, BxPC3, A2780 and A2780 cisR cells; ii) F-12 HAM'S (Sigma Chemical Co.) for A549 and PC3 cells; iii) D-MEM medium (Euroclone) for A375 cells; iv) EMEM for A498 cells.

Cytotoxicity MTT assay. The growth inhibitory effect towards human cell lines was evaluated by means of MTT (tetrazolium salt reduction) assay.[1] Briefly, 3-8·10^3 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 additional incubation, 100 μL of a sodium dodecylsulfate (SDS) solution in HCl 0.01 M were added. After overnight incubation, the inhibition of cell growth induced by the tested complexes was detected by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, CA). Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted vs drug concentration. IC_{50} values represent the drug concentrations that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells.

Cellular accumulation. MCF-7 cells (2.5×10^6) were seeded in 75 cm^2 flasks in growth medium (20 ml). After overnight incubation, the medium was replaced and the cells were treated with tested compounds for 24 h. Cell monolayers were washed twice with cold PBS, harvested and counted. Cell nuclei were isolated by means of the nuclei isolation kit Nuclei EZ Prep (Sigma Co.). Samples were than 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 μg 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. MCF-7 cells (3×10^6) were seeded in 10 cm Petri dishes in 10 mL of culture medium. Subsequently, cells were treated with 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 artifacts. The samples were completely dried and re-dissolved in 200 μL of Milli-Q water (18.2 MΩ) for at least 20 min at 65 °C in a shaking thermo-mixer, mineralized and analyzed for total Pt content by GF-AAS as described above.

Histone deacetylase assay. Histone deacetylase activity was determined using Fluor-de-Lys® HDAC fluorometric activity assay kit (Enzo Life Sciences International, Inc., Plymount Meeting, PA, U.S.A.). MCF-7 cells (5·10^4 seeded in 96-well microplates) were treated for 24 h with equi-toxic concentrations of tested complexes, corresponding to the IC_{50} values, and then processed as reported by the manufacturer's instructions. Fluorescence was measured using a Fluoroskan Ascent FL (Labsystem, Finland) plate reader, with excitation at 360 nm and emission at 460 nm. For comparison purpose, the HDAC activity was also measured in nuclear extracts of MCF-7-treated cells by means of the same fluorometric activity assay kit. In this latter case, MCF-7 nuclear extracts were obtained as follow: cell pellets were re-suspended in lysis buffer [Tris·HCl (10 mM, pH 8.0), KCl (60 mM), EDTA (1.2 mM), DTT (1 mM), PMSF (0.05 mM), NP-40 (0.05%)] and kept on ice for 10 min. Subsequently, samples were centrifuged at 1000g and re-suspended in the nuclear extraction buffer

[Tris·HCl (20 mM, (pH 8.0), NaCl (420 mM), MgCl2 (0.7 mM), EDTA (0.25 mM), glycerol (25%)] for 30 min at 4 °C, and then centrifuged at 15000g for 15 min at 4 °C.

Caspase-3/-7 activation. Caspase-3/-7 activity was detected by using the Apo-One® 3/7 Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) according to the manufacturer's recommended procedures. MCF-7 cells were seeded in 96-well microplates in growth medium (100 µL) and then incubated at 37°C in a 5% carbon dioxide atmosphere. After overnight incubation, cells were subjected to a 24 h treatment with tested compounds (at concentrations corresponding to IC_{50} values). Subsequently, each well was treated with 120µl of the Apo-ONE® Caspase-3/7 Assay Reagent containing the specific substrate (rhodamine 110 bis-(N-CBZ-l-aspartyl-l-glutamyl-l-valyl-aspartic acid amide), Z-DEVD-R110). The fluorescence was determined after one hour with a PerkinElmer 550 spectrofluorometer (excitation 499 nm, emission 521 nm).

Hoechst 33258 staining. MCF-7 cells were seeded into 8-well tissue-culture slides (BD Falcon, Bedford, MA, USA) at $5 \cdot 10^4$ cells/well (0.8 cm^2). After 24 h, cells were washed twice with PBS and following 72 h of treatment with IC_{50} concentration of tested complexes, cells were stained for 5 min with 1 mg/mL Hoechst 33258 (Sigma–Aldrich) in PBS before being examined by fluorescence microscopy (Olympus BX41, Cell F software, Olympus, Munster, Germany).

Nuclear DNA fragmentation. MCF-7 cells (10^4/well) were treated in a 96-well plate at 37 °C for 12 and 24 h with IC_{50} doses of tested compounds. Afterwards, the plate was centrifuged for 10 min, the supernatant removed, and the pellet treated according to the manufacturer's instructions of ELISAplus cell death detection kit (Roche). The extent of nuclear fragmentation was measured in a plate reader following absorbance at 405 minus 492 nm using a fluorescence microplate reader (Fluoroskan Ascent FL, Labsystem, Finland).

Western blot analyses. About 10^6 MCF-7 cells were treated for 24 h with IC_{50} doses of tested compounds. Afterwards, cells were harvested and lysed in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS), and were centrifuged at 13,000xg for 15 min at 4°C. β-actin was used as a loading control. An equal amount of proteins for each sample was electrophoresed on a 12% SDS-PAGE and blotted to a nitrocellulose membrane. The membrane was incubated for 1 h in PBS-Tween20 (0.05%) containing 5% nonfat milk and then at 37°C for 1 h with primary antibodies. The membranes were stained with the corresponding peroxidase-conjugated secondary antibodies for 1 h at room temperature and detected by ECL according to the manufacturer's protocol (GE).

Mitochondrial membrane potential (ΔΨ). ΔΨ was assayed using the Mito-ID Membrane Potential Kit according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY). Briefly, MCF-7 cells were seeded onto 96-well microplates at 5 x 10^4 cells/well. After 24 h, cells were treated with tested compounds for 24 or 48 h. The mitochondrial depolarizing agent, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was employed as positive control. An equal volume of cationic dye loading solution was added to each well and cell plates were incubated for additional 30 min at 37°C. Plates were read at excitation/emission wavelengths 490 and 590 nm using a fluorescence microplate reader (Fluoroskan Ascent FL, Labsystem, Finland).

Cell lines and culture for the studies of p53 dependence

The human colon carcinoma HCT-116 (parental, $p53^{+/+}$) and its p53-null derivative ($p53^{-/-}$) cells[5] were a kind gift of Dr. M. Brazdova, Institute of Biophysics, Brno (Czech Republic). HCT116 $p53^{+/+}$, and HCT116 $p53^{-/-}$ cells were grown in DMEM supplemented with streptomycin (100 µg mL^{-1}), penicillin (100 U mL^{-1}), and heat inactivated FBS (10%). The cells were cultured in a humidified incubator at 310 K in an atmosphere of 5% CO_2 and subcultured 2−3 times per week with an appropriate plating density. HCT-$116^{+/+}$, $^{-/-}$ were seeded at the density 4000 cells/well.

Involvement of p53 pathway in the mechanism of action of I and VI

The effect of p53 status on the cytotoxic activity of compounds I and IV was tested on two isogenic human colon cancer cells, wild-type p53-containing HCT-116 cells (parental cells ($p53^{+/+}$) and its p53-null derivative ($p53^{-/-}$)). Briefly, HCT-116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were seeded in 96-well black plates (Corning) at the density 4000 cells/well in 100 μL of growth medium (DMEM) and incubated overnight (16 h). Then the cells were treated with compounds I and VI and incubated for 72 h at 310 K in 5% CO_2 humidified atmosphere. DMF-supplemented DMEM was introduced to the untreated controls. After the incubation period, the medium was replaced with 0.1% Triton X-100 in MQ water. The cells were lysed under continuous shaking on ice for 30 min. Subsequently, the cells were freeze−thawed for five cycles. An equal volume of 2×SYBR Green I was added to 100 μL of the lysate. DNA contents were measured with fluorescence reader Varian Cary Eclipse at excitation/emission wavelength 485/535 nm. Experiments were done in triplicate. The data were normalized to the DNA content in control, DMF treated cells.

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[1] M. C. Alley, D. A. Scudiero, A. Monks, M. L. Hursey, M. J. Czerwinski, D. L. Fine, B. J. Abbott, J. G. Mayo, R. H. Shoemaker and M. R. Boyd, *Cancer Res*, 1988, **48**, 589-601.