Supporting Information

Increased immune cell infiltration in patient-derived tumor explants treated with Traniplatin: an original Pt(IV) pro-drug based on Cisplatin and Tranilast

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Materials and Methods.

Tranilast was purchased from TCI Europe and used as supplied. NHS (Hydroxysuccinimide), DCC (N,N'-Dicyclohexylcarbodiimide) and K_2CO_3 were purchased from Sigma Aldrich. Cisplatin and Oxoplatin were synthesized as previously described [1, 2].
All the other chemicals and solvents were of analytical or spectroscopic grade, obtained from commercial sources and used without further purification. Deuterated solvents were obtained from Apollo Scientific.

Elemental analyses (carbon, hydrogen and nitrogen) were performed with a PerkinElmer 2400 series II analyzer.

ESI Mass Spectra were recorded with a Waters LCT Premier XE Spectrometer.

ICP-MS: Platinum determination was performed with an Agilent 7500ce Series Inductively Coupled Plasma-Mass Spectrometer.

NMR: ^{1}H and ^{13}C spectra were obtained in solution of $CDCl_3$ or DMSO-d_6 and ^{195}Pt NMR spectrum was obtained is DMF solution (Insert of D_2O) at 300 K, in 5-mm sample tubes, with an Agilent Varian 500 MHz premium shielded (operating at 500.13, 125.75 and 107.49 MHz respectively). The ^{1}H chemical shift was referenced to the residual impurity of the solvent. The external references was Na_2PtCl_4 in D_2O (adjusted to δ = -1628 ppm from Na_2PtCl_6) for ^{195}Pt.

HPLC Studies. The purity and the stability of TPT was assesses using HPLC. HPLC chromatograms were obtained on a Waters Alliance 2695 with an automatic injector and a photodiode array detector 2998 Waters (Waters, Milford, MA) using a Sunfire C18 column (100 x 4.6 mm x 5 µm, 100 A, Waters) and software EmpowerPro 2. The flow rate was 1 mL/min using MeCN (1% TFA) and H_2O (1% TFA). Gradient G0100 were used in all cases. Traniplatin was dissolved in DMF (0.5 ml) and diluted to a final concentration of 0.5 mM using acetonitrile and water solution (1/1).

The reduction of the Traniplatin with ascorbic acid was followed *via* high-performance liquid chromatography (HPLC) using a Phenomenex Luna C18 (5 µM, 100 Å, 250 mm × 4.60 mm i.d.) column at RT at a flow rate of 1.0 mL/min with 254 nm UV detection. Mobile phase containing 80:20 acetonitrile (1% trifluoroacetic acid): water (1% trifluoroacetic acid). Traniplatin was dissolved in DMF (0.5 ml) and diluted to a final concentration of 0.5 mM using acetonitrile and water solution (1/1) with 5 mM ascorbic acid and 2 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7). The process was followed at 37 °C until complete reduction. The HPLC chromatogram from the reduction reaction is compared with the chromatogram of pure samples of the tranilast.

Log P_{ow} Determination. The Log P_{ow} determination of the traniplatin was conducted using the shake flask method [3]. Traniplatin was dissolved in 0.9% NaCl w/v ultrapure water (presaturated with n-octanol for 96 h and left to stand overnight). The solutions were sonicated and filtered through Celite to remove undissolved traniplatin. The initial concentrations of platinum content were determined by ICP-MS. Subsequently, the platinum(IV) solutions were added an equal volume of *n*-octanol (presaturated with 0.9% NaCl w/v ultrapure water for 96 h and left to stand overnight). The heterogeneous mixtures were shaken vigorously for 2 h before centrifuging for 15 min to achieve phase separation. The final concentration of platinum content in the aqueous phase was determined again by ICP-MS, and the water-octanol partition coefficient was calculated. The experiment was done in triplicates.

Cell Culture. A549 (non-small lung cancer, human), HT29 (colon adenocarcinoma, human),Hela (cervix adenocarcinoma, human), RAW 264.7 (macrophages, mouse) cells were grown in 75 cm^2 culture flasks (Corning® Flask) as adherent monolayer cultures in Dulbecco´s Modified Eagle Medium (DMEM) (GIBCO, ref. 41966-029). PC-3 and C4-2 prostate cancer cells were grown in 75 cm2 culture flasks (Corning® Flask) as adherent monolayer cultures in RPMI medium 1640 (GIBCO, ref. A10491-01). Both culture mediums were supplemented with 10% heat-inactivated fetal bovine serum, and with Penicillin/Streptomycin (Gibco, 15140-122). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Inhibition of Cell Viability Assay. Drug effects on exponentially growing tumour cells were determined using XTT assay as described previously. [4] PC3, C4-2, HT29 and A549 were seeded at a density of 3000 cells/well in 96-well plates and incubated for 24h. Thereafter, cancer cells were exposed to drugs at different concentrations during 72h. At 72h, 50 µL of XTT/ECR 1:50 solution (ROCHE ref.: 11465015001) and incubated for a further 4 h at 37 ^oC. Absorbance measured at 475 nm was converted to percentages. UV-vis absorbance was measured at 475 nm using a microplate reader (PowerWave$_X$, BIO-TEK). Experiments were performed in triplicated for each drug concentration and carried out independently at least three times. The interpolation analysis was done using dose-dependent inhibition pattern with Prism version 5.00 software (GraphPad Software, USA). The statistical analysis was done using Prism version 5.00 software (GraphPad Software, USA). The paired t-test (two-tailed) was applied.

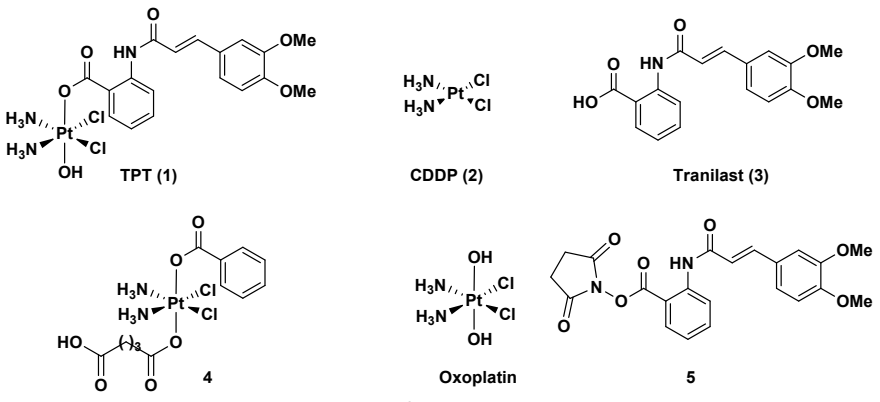
Apo-ONE Homogeneous Caspase-3/7 Assay. The homogeneous caspase-3/7 assay kit (Promega, Madison, WI), which provides a profluorescent substrate with an optimized bifunctional cell lysis/activity buffer for caspase-3/7 activity was used. A549 and HT29 cells were seeded at a density of 3000 cells/well in a black 96-well plate and incubated for 24h. Thereafter, cancer cells were exposed to 10 µM of **1** or **2** for 24h. The apo-ONE homogeneous caspase-3/7 assay was performed according to the manufacturer's protocol. The buffer and the substrate were mixed and added to the samples. The excitation/emission (483 Ex/525 Em) was recorded on SAFIRE^2 with XFLUOR4SAFIREII Version: V 4.62n software.

Platinum Uptake Experiment. Platinum determination was performed with an Agilent 7500ce Series Inductively Coupled Plasma-Mass Spectrometer. For quantitative determination the most abundant isotopes of platinum were measured at m/z 195. A549 and HT29 cells were seeded at a density of 200000 cells/well in a 6-well plate and incubated for 24h. Thereafter, cancer cells were exposed to 10 µM of compound **1** or **2** for 4h. After incubation, cells were washed with PBS followed by trypsinization. The cell suspension was centrifuged at 1000 rpm for 5 min at 4 °C and the pellets were stored at -80 °C. The pellets were suspended in 300 µL of mQ H_2O and sonicated during 30 minute. Protein concentration was determinate using the BCA protein assay reagent (Thermo Scientific catalogue number: 23225) according to the manufacturer's instructions (micro-well plate protocol). The remaining cell suspension was transferred into a Teflon reactor with 300 µL of HNO_3 65% and mineralized at 90 °C for 18h. The samples were diluted with mQ H_2O until 2% HNO_3 concentration was reached. Platinum determination was performed with ICP-MS.

Human tumor explants. Freshly resected tumor samples from colorectal cancer patient collected at Hospital del Mar were were sliced in equivalent pieces, maintained in culture at 37ºC, 5% CO_2 and treated ex vivo with traniplatin (TPT, 10 µM) or Cisplatin (CDDP, 10 µM) during 72h. Explants were cultured in patient-derived CoSC-specific media - Advanced DMEM/F12; 10 mM HEPES; 1x Glutamax; 1x B-27 without retinoic acid; 1x N-2; 1 mM N-Acetyl-L-cysteine and 50 ng/mL EGF [4]. Samples were treated in vitro for 72h and compared to non-treated control. Following in vitro treatment, samples were processed for further pathological analysis -tissue fixation, inclusion and sectioning. Following treatment, samples were processed for histopathological analysis. Immunohistochemical staining for leukocytes (CD45) and apoptotic cells (cleaved Caspase-3) in the biopsy specimens was performed at IRB Histopathology core facility. Immunostainings were carried out on 4-µm tissue sections using antibodies against CD45 (Dako, IS751) or cleaved Caspase-3 (Cell Signalling, 9661S) according to standard procedures. Qualitative evaluation was performed by expert pathologist (M.I.) in a blinded fashion with respect to experimental settings. Presence of CD45 (+) or cleaved Caspase-3 (+) cells in high-power field (HPF) was determined for each tumor sample using light microscopy from randomly selected HPFs in the tumor sample.

Ethical approval. Samples are obtained under informed consent and approval of the Tumor Bank Committee according to Spanish ethical regulations (Ley Orgánica 15/1999 de diciembre de Protección de Datos de carácter personal –LOPD-). The study follows the guidelines of the Declaration of Helsinki, and patient identity for pathological specimens remains anonymous in the context of this study.

Synthesis



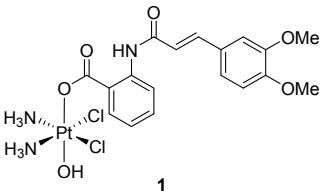
Scheme S1

2,5-dioxopyrrolidin-1-yl (E)-2-(3-(3,4-dimethoxyphenyl) acrylamido) benzoate (5). Tranilast (**3**) (0.4 g, 1.22 mmol) and NHS hydroxysuccinimide (0.14 g, 1.21 mmol) were dissolved in 25 mL of dry THF

and DCC, dissolved in dry THF (10 mL, 122 mM), was added dropwise. A white solid immediately precipitated and the reaction mixture was stirred at RT overnight. The white solid obtained was filtered-off and the yellow solution was dried under vacuum to give a yellow solid that was re-dissolved in 20 mL of dichloromethane and kept at 4°C overnight. Another small portion of white solid was formed and removed by filtration. The yellow solution was dried under vacuum obtaining **5** as a yellow solid (321 mg, 62%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.20 (dd, 1H), 7.80 (d, 1H), 7.79 (d, 1H), 7.61 (d, 1H), 7.48 (dd, 1H), 7.18 (d, 1H), 7.16 (s, 1H), 6.90 (d, 1H), 6.71 (d, 1H), 3.94 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 2.74 (s, 4H, NHS). HRMS-ESI calcd. for $C_{22}H_{11}O_7N_2$ 425.1343 found 425.1354 $[M+H]^+$

Traniplatin (1). Oxoplatin (65 mg, 0.194 mmol) and tranilast-NHS (**5**) (75 mg, 0.177 mmol) were suspended in 6 mL of dry DMSO and the mixture was stirred at 60°C for 15 h. The yellow residue (oxoplatin in excess) was filtered off and the yellow solution was lyophilised overnight obtaining a sticky yellow solid. The solid is dissolved in 4 mL of DMF and precipitated by addition of Et_2O. The solid was washed 3 times with 5 mL of dichloromethane and 2 times with 5 mL of Et_2O and dried under vacuum overnight giving **1** as a yellow solid (37 mg, 0.057 mmol, 33%). Anal. Calcd. for $C_{18}H_{23}Cl_2N_3O_6Pt$: C 33.60, H 3.60, N: 6.53, found C 33.84, H 3.42, N 6.76. ^{1}H NMR (DMSO-d_6, 500 MHz): δ = 11.33 (s, 1H), 8.71 (dd, 1H, *J*=8.4, 1.2 Hz), 7.94 (dd, 1H, *J*=7.9, 1.7 Hz), 7.52 (d, 1H, *J*=15.6 Hz), 7.51-7.47 (m, 1H), 7.44 (d, 1H, *J*=2.0 Hz), 7.23 (dd, 1H, *J*=8.3, 1.9 Hz), 7.14 (d, 1H, *J*=15.6 Hz), 7.09 (dd, 1H, *J*=7.40, 7.27 Hz), 7.00 (d, 1H, *J*=8.3 Hz), 6.39-5.86 (m, 6H), 3.87 (s, 3H), 3.80 (s, 3H) ppm; ^{13}C NMR (DMSO-d_6, 125.75 MHz) δ 172.7 (*C*), 164.4 (*C*), 150.9 (*C*), 149.5 (*C*), 140.8 (*C*H), 140.2 (*C*), 132.7 (*C*H), 132.0 (*C*H), 128.1 (*C*), 123.1 (*C*H), 122.5 (*C*H), 121.7 (*C*H), 121.3 (*C*), 119.7 (*C*H), 112.0 (*C*H), 110.1 (*C*H), 56.4 (CH_3), 56.0 (CH_3) ppm; ^{195}Pt NMR (107.49 MHz DMF insert D_2O): δ = 985 ppm. HRMS-ESI calcd. for $C_{18}H_{24}O_6N_3Cl_2Pt$ 643.6084 found 643.0692 $[M+H]^+$

Figure S1. ^{1}H-NMR of Traniplatin (1) in DMSO-d_6



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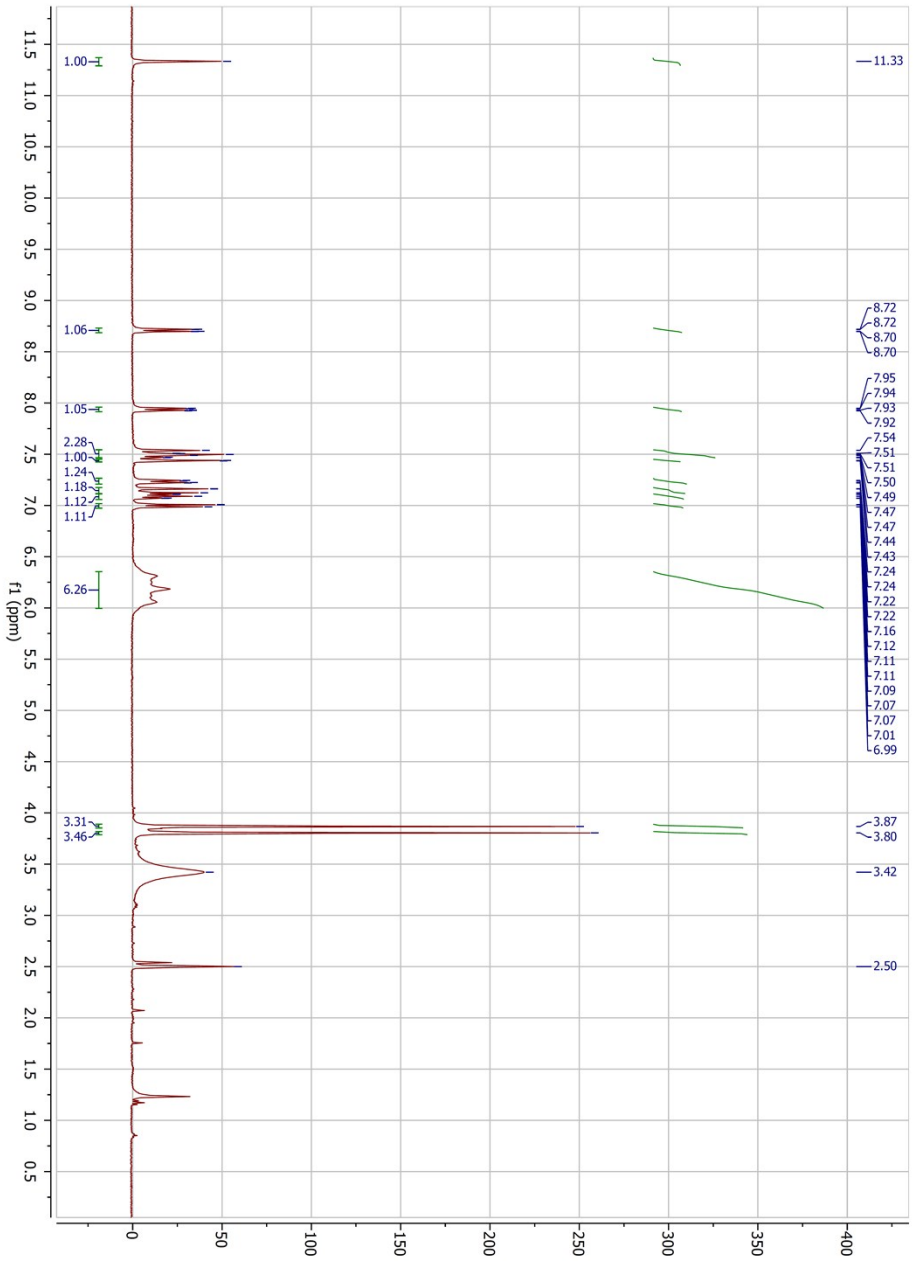
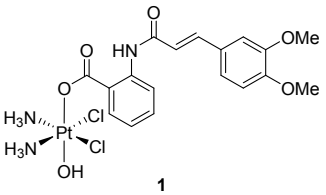


Figure S2. ^{13}C-NMR of Traniplatin (1) in DMSO-d_6



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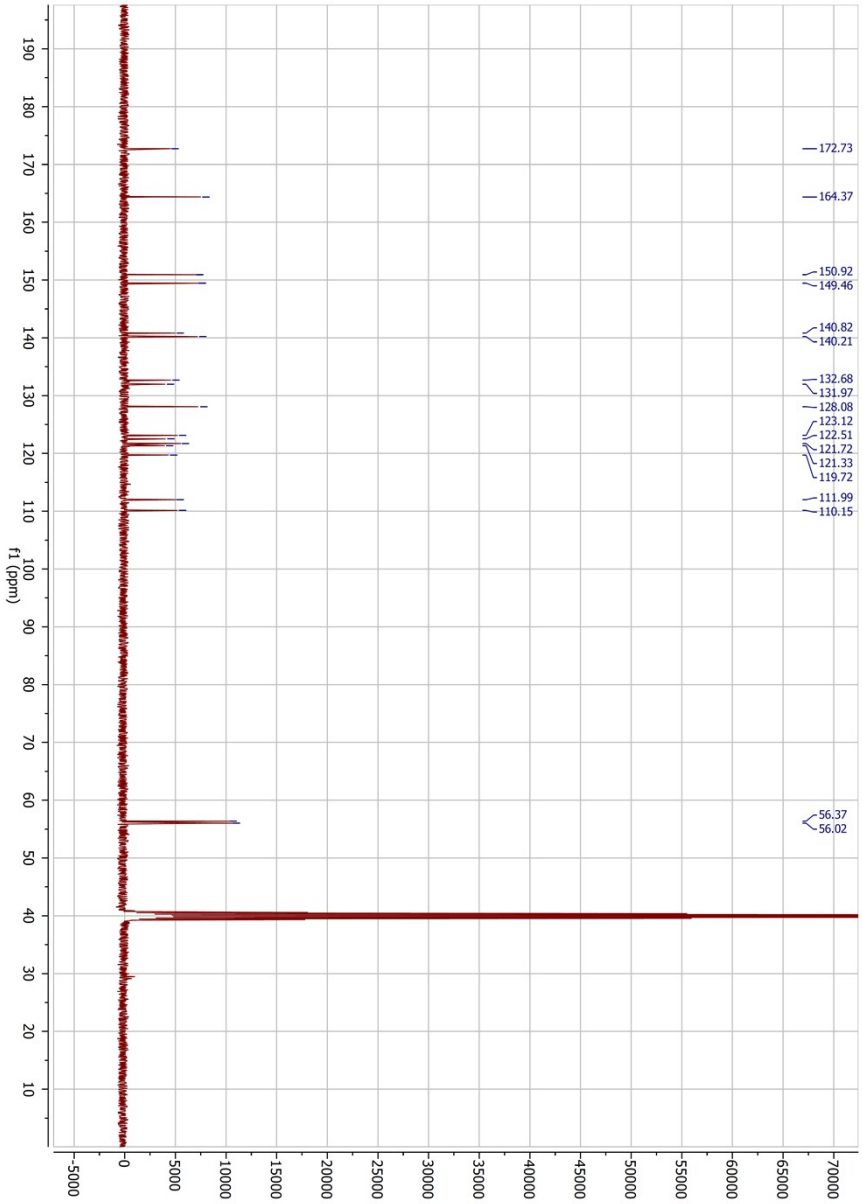


Figure S3. COSY-NMR of Traniplatin (1) in DMSO-d_6

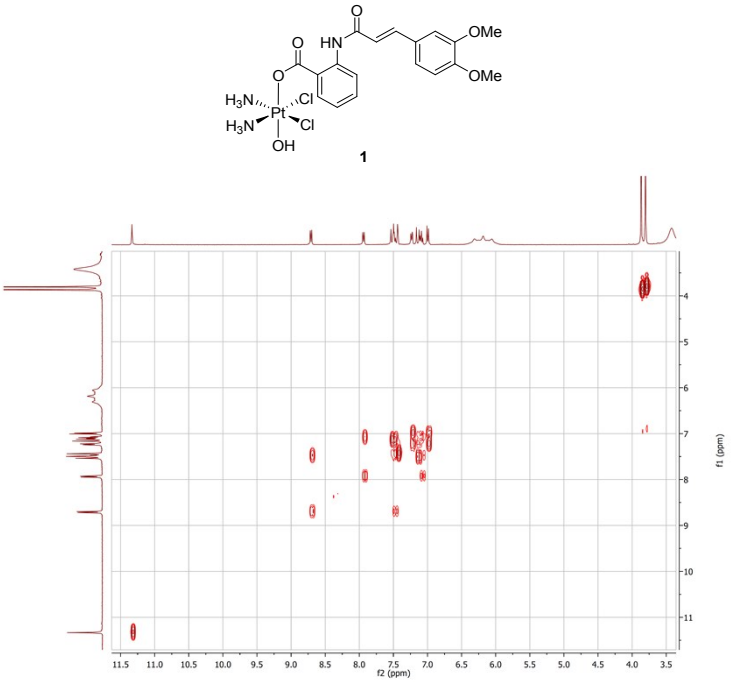


Figure S4. HSQC-NMR of Traniplatin (1) in DMSO-d_6

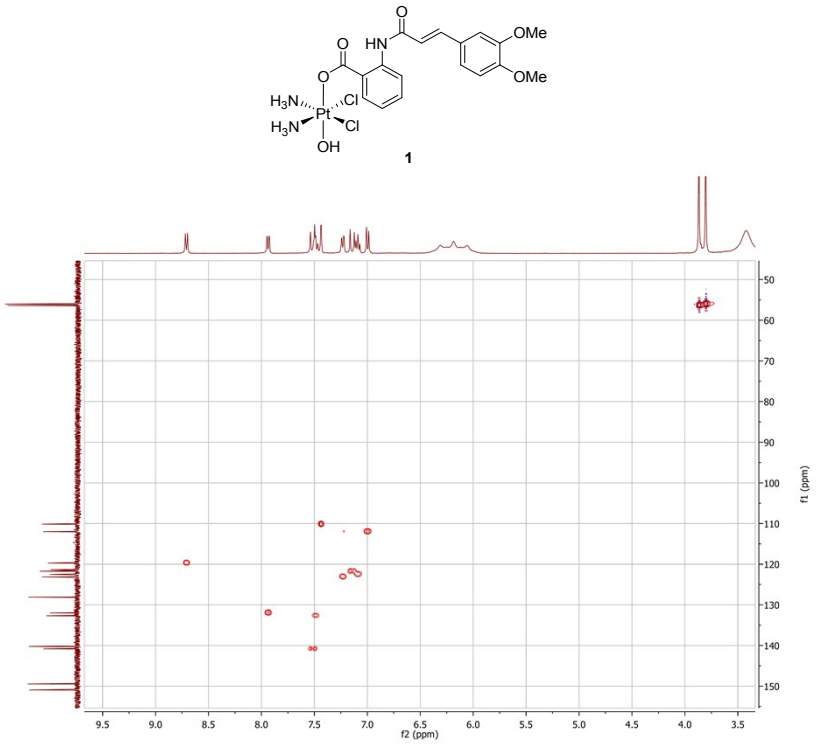


Figure S5. ^{1395}Pt-NMR of Traniplatin (1) in DMF (D_2O)

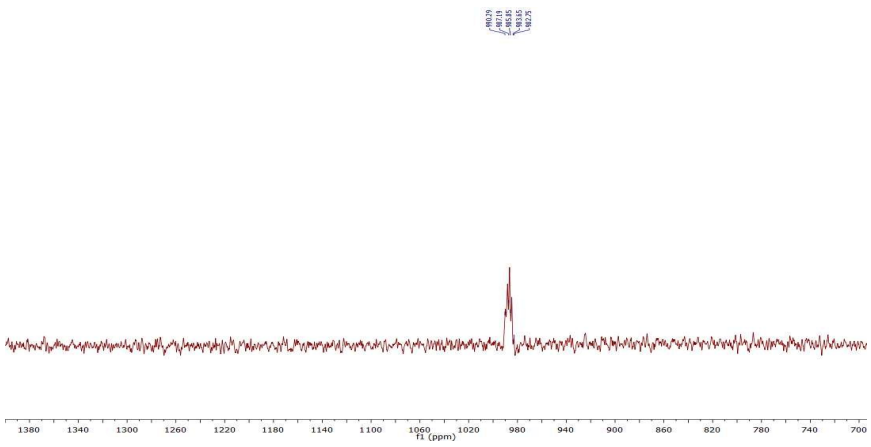
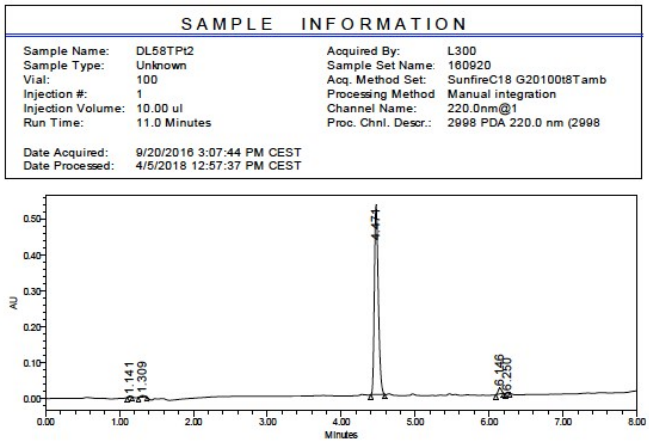


Figure S6. HPLC chromatogram of Traniplatin using a Sunfire C18 (mobile phase H_20 1% TFA and ACN 1% TFA).



	RT	Area	% Area	Height
1	1.141	6246	0.31	2518
2	1.309	17206	0.84	4289
3	4.471	1963010	96.07	528019
4	6.146	50239	2.46	15893
5	6.250	6570	0.32	2924

Figure S7. HPLC chromatogram showing : a) traniplatin (**1**) + Ascorbic Acid (AA) at t= 0 ; b) traniplatin **1**+AA *t* =1h; c) **1**+AA *t* =6h; d) **1**+AA *t* =10h; e) **1**+AA *t* =20h; f) **1**+AA *t* =30h; g) tranilast (**3**). * is AA.

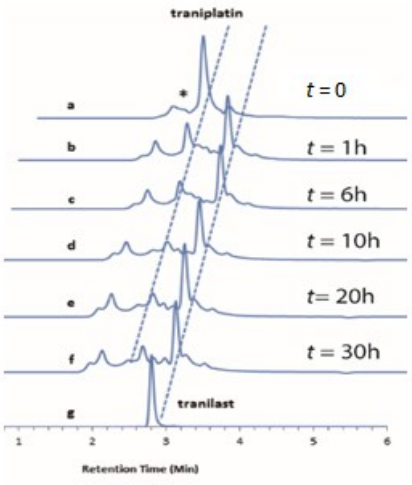


Figure S8. HPLC chromatogram of Traniplatin at t=0 (blue line), Tranilast (green line) and Traniplatin at t=24h in H_2O/ACN (red line).

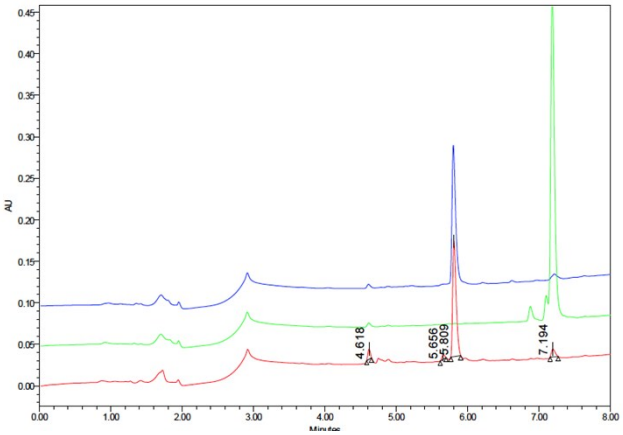
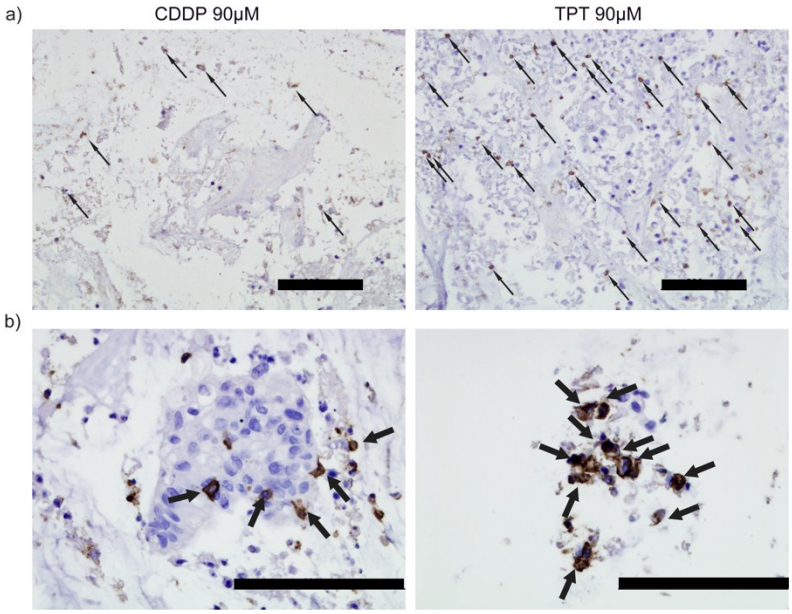


Figure S9. a-b) Cell membrane CD45 reactivity in representative tumor explants treated *ex vivo* with CDDP 90 µM (left panels) or TPT 90 µM (right panels) for 72h; arrows point to positive -immune-cells in the tumor. Scale bars, 200 µm. a) Explants treated with TPT showed overall occasional presence of CD45+ (right panel) compared to CDDP-treated tumor explants (left panel). b) left panel: a hotspot of 5 CD45+ cells detected in explant treated with CDDP, right panel: a hotspot of 10 CD45+ cells explants detected in TPT treated explant.



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[2] M. Galanski, B. K. Keppler, *Inorg. Chem.* 1996, **35**, 1709-1711.

[3] Test No. 107: Partition Coefficient (n-octanol/water): Shake Flask Method; OECD Publishing: Paris, 1995.

[4] A. Calon et al, Cancer Cell, **2012**, 22, 571-584; A. Calon et al, *Nature Genetics*, **2015**, *47*, 320-329