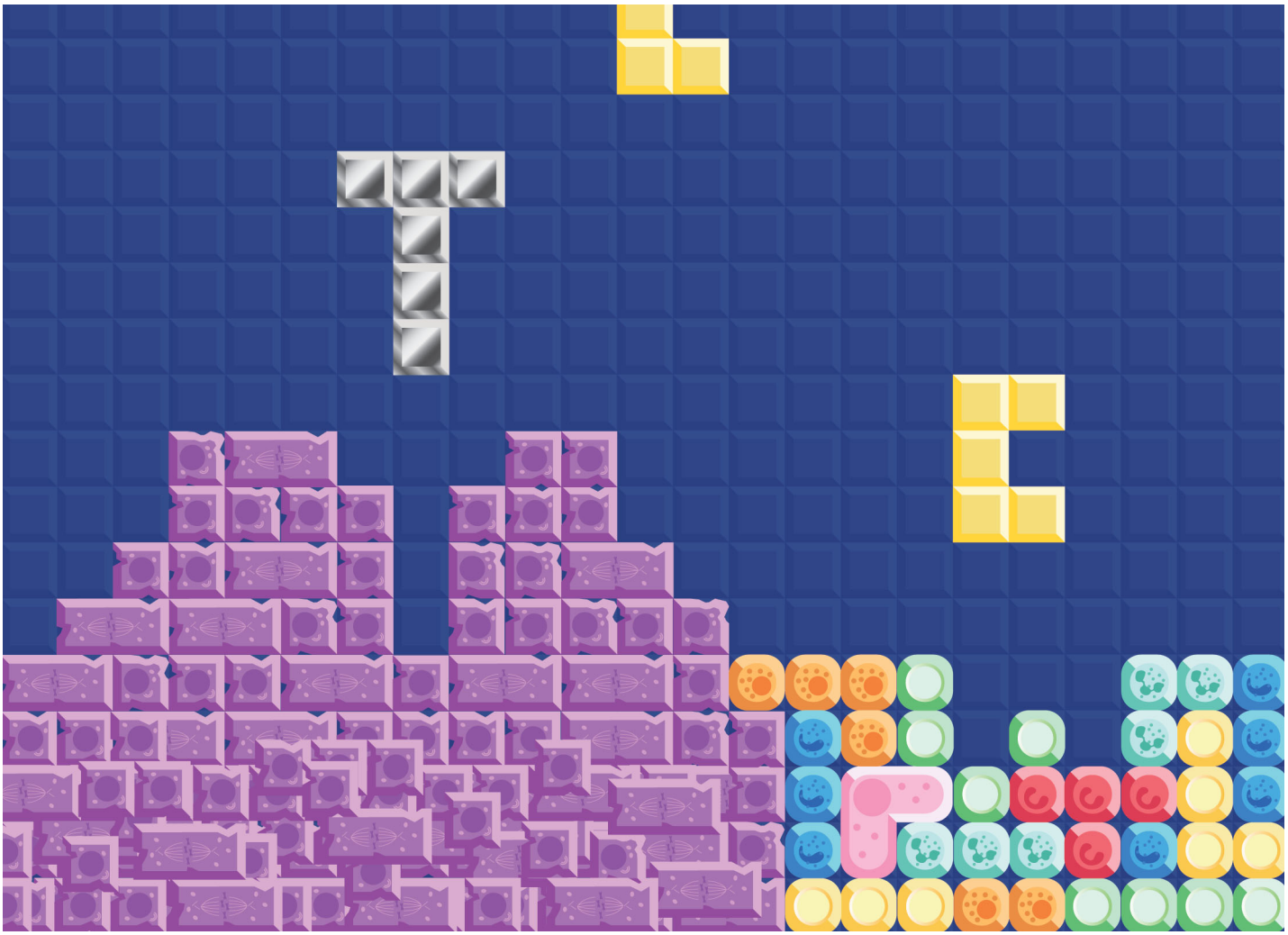

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Increased immune cell infiltration in patient-derived tumor explants treated with Traniplatin: an original Pt(IV) pro-drug based on Cisplatin and Tranilast†

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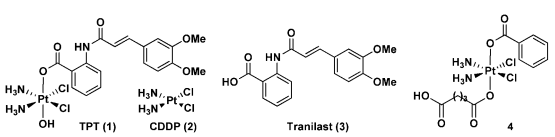
Elevated intra-tumoral immune infiltrate is associated with an improved prognosis in cancer of distinct origins. Traniplatin (TPT) is a novel platinum(IV) pro-drug based on Cisplatin (CDDP) and the marketed drug Tranilast. When compared *in vitro* to Cisplatin, TPT showed increased cytotoxic activity against colon and lung cancer cells but decreased activity against immune cells. In addition, TPT efficiency was evaluated in tumor explants derived from colorectal cancer samples from patients subjected to intended curative surgery. TPT induced strong intra-tumoral cytotoxic activity yet was associated with an elevated presence of immune cell infiltrate, suggesting a reduced cytotoxic activity against immune cells in colorectal cancer.

Colorectal cancer (CRC) is considered to be the third most common cancer and the fourth leading cause of cancer death worldwide.[1] 5-FU in combination with oxaliplatin (FOLFOX) represents the standard of care for CRC. However, many patients do not respond to FOLFOX or only show a marginal improvement.[2] Hence, CRC incidence and mortality are predicted to increase unless new therapies are discovered.[3] CDDP (Cisplatin **2**, Scheme 1) is commonly used for the treatment of several cancers including colon,[4] testicular, ovarian, lung, head and neck.[5,6] The benefit of platinum therapies is often counterbalanced by the appearance of severe side effects.[5] Thus, the development of stable platinum Pt(IV) complexes that can mitigate some of these limitations has gained significant attention. Pt(IV) species could overcome the high toxicity of Pt(II)-based drugs[7,8] due to increased stability compared to the corresponding Pt(II) species.[9] In addition, Pt(IV) complexes can get to the cancer cellular environment without reacting with sulfur-containing biomolecules, which are the main cause of nephrotoxicity.[10] One example is Satraplatin, an orally administrated Pt(IV) complex that has reached phase III trials.[11] Pt(IV) pro-drugs are reduced inside the cell to the corresponding Pt(II) species, with the release of the axial ligands.[12] The intracellular reduction has been demonstrated by XANES spectroscopy and fluorescence techniques.[13–15] It has been reported that the physicochemical properties of Pt(IV) complexes, such as aqueous solubility or lipophilicity, can be tuned by modification of the axial substituents.[16] Tranilast is commercialized as an anti-allergy drug used for the treatment of inflammatory diseases.[17] Tranilast (**3**) also reduces pathological fibrosis associated with myocardial infarction,[17] inhibits human breast cancer cell migration[17] and has raised interest for its properties as a cytostatic agent in prostate, breast and pancreatic cancer as well as in glioma and other tumors.[18] Furthermore, Tranilast is a relatively safe drug with modest side effects. At doses of 600 mg day^{-1}, it is well tolerated by patients over a period of months.[17] The mechanism by which Tranilast acts as an anti-proliferative drug is still debated. Some data suggest that it suppresses the TGF-β pathway.[17,18] However, other mechanisms have been proposed[17] such as inhibition of MMP-2/9 production, inhibition of the EMT and suppression of the activation of NF-κB, protein kinase C (PKC) and MAPKs.

Here we report the synthesis, characterization and a deep biological evaluation of Traniplatin (TPT), a new Pt(IV) pro-drug with Tranilast in the axial position (Scheme 1).

Tranilast (**3**) was successfully conjugated to CDDP (**2**) by reacting oxoplatin[19] (**4**) with the NHS-ester derivative of Tranilast (**5**)



Scheme 1 Traniplatin (TPT, **1**), Cisplatin (CDDP, **2**), Tranilast (**3**) and compound **4**.

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(see Synthesis, ESI†). The new Pt(IV) derivative TPT (**1**) was characterized using elemental analysis, ^{1}H, ^{13}C and ^{195}Pt NMR, and HPLC (Fig. S1–S6, ESI†). Unfortunately, attempts to synthesize the bis-adduct were unsuccessful. Since the mechanism of action of Pt(IV) pro-drugs requires their intracellular reduction to the corresponding Pt(II) species with release of the axial ligands, TPT reduction was studied *via* HPLC and it was completely reduced by ascorbic acid with Tranilast release in 30 hours (Fig. S7, ESI†). TPT is stable for up to 24 h in aqueous/ACN solution (Fig. S8, ESI†).

Biological activity. The sensitivity of cancer cells to the newly synthesized TPT was evaluated on a panel of different cancer cell lines, such as in human-originating prostate (PC-3 and C4-2), cervical (HeLa), colorectal (HT29) and lung (A549) cancer cell lines. TPT efficiency was compared to CDDP, Tranilast (**3**) and to a mixture (1 : 1 ratio) of CDDP and Tranilast (**3**). Prostate and cervical cancer cells where treated with increasing concentrations of TPT, demonstrating cytotoxic activity in line with that of CDDP (Fig. 1a).

Interestingly, TPT was found to be 4-fold more potent than CDDP in lung cancer cells and 10 times more cytotoxic than CDDP in CRC cells, suggesting a specific affinity of TPT for lung and colon cancer cells (Fig. 1a). Of note, the free axial ligand Tranilast (**3**) only showed weak activity against all of the treated cancer cell lines in our experimental setting. We next analyzed the capacity of TPT to induce cancer cell death. To this end, caspase-3/7 activation – an essential step of apoptosis – was measured in treated cancer cells. An optimal experimental setting was defined for each cancer cell line. As shown in Fig. 1b, incubation of lung cancer cells (A549) and colon cancer cells (HT29) for 24 h cells with TPT (10 μM) resulted in a significantly higher activation of caspase-3/7 compared to that of the CDDP treatment. Increased accumulation of an active platinum species may result in enhanced cytotoxicity. Cellular uptake of a platinum drug could be influenced by the overall lipophilicity of a specific platinum drug. It has been described previously that the introduction of simple ligands[20] results in increased platinum cellular-uptake associated with an enhanced cytotoxic activity. The lipophilicity index $\log P_{ow}$ value determined for TPT was -0.41 ± 0.09, which is in line with previous data reported for similar compounds[16] and is higher than the $\log P_{ow}$ of CDDP (-2.03 ± 0.47).[16] Since the higher lipophilicity of TPT could explain its enhanced biological activity,[16] we speculated that a Pt(IV) compound with a similar $\log P_{ow}$ may show a comparable biological activity. We therefore synthesized compound **4** (Fig. 2) which is reported to have a

a)

IC_{50} (μM)	PC-3	C4-2	HeLa	HT29	A549
TPT (1)	4.3 ±1.1	4.1 ±0.7	4.2 ±0.6	4.7 ±0.8	0.5 ±0.1
CDDP (2)	3.9 ±0.4	3.1 ±1.5	8.1 ±0.8	45.7 ±2.5	2.0 ±0.3
Tranilast (3)	37.4 ±4.0	>100	72.7 ±4.1	>100	69.6 ±15.5
2 + 3 (1:1)	2.9 ±0.2	5.7 ±0.7	7.1 ±0.6	54.7 ±5.1	2.1 ±0.4

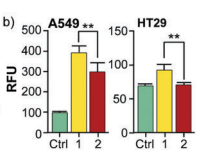

Fig. 1 (a) IC_{50} was determined after 72 h treatment in the presence of TPT (**1**), CDDP (**2**), Tranilast (**3**) and CDDP/Tranilast (1 : 1 ratio) the indicated cancer cell lines; (b) caspase-3/7 activation as a result of Pt-based drugs treatment at 10 μM concentration of TPT (**1**) or CDDP (**2**) for 24 h; unpaired *t*-test (two-tailed) was applied. $^{**}p < 0.01$.

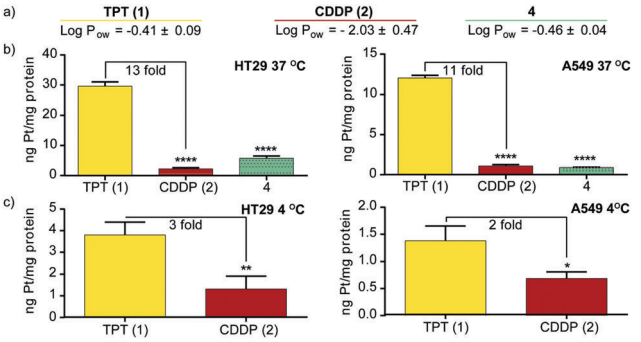

Fig. 2 (a) $\log P_{ow}$ of TPT (**1**), CDDP (**2**), and **4**; (b) platinum uptake in lung (A549) and colorectal (HT29) cancer cells after TPT (**1**), CDDP (**2**), or compound **4** treatments at 37 °C; (c) platinum uptake in lung (A549) and colorectal (HT29) cancer cells after TPT (**1**), CDDP (**2**), treatments at 4 °C. Cells were incubated with 10 μM TPT (**1**), CDDP (**2**), or compound **4** for 4 h. Platinum levels in the cancer cells were measured using ICP-MS. $^{****}p < 0.0001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

$\log P_{ow}$ comparable to that of TPT (-0.46 ± 0.04 *vs.* -0.41 ± 0.09 respectively).[16] Surprisingly, compound **4** was less cytotoxic than TPT in A549 and HT 29 cells [compound **4**: IC_{50} = 6.3 μM (A549) and 34.8 μM (HT29) *vs.* TPT: 0.5 μM (A549) and 4.7 μM (HT29)]. The reduced activity of **4** could be explained by a decreased Pt(IV) internalization together with a slow reduction rate and a reduced release of the cytotoxic Pt(II) species.

We therefore investigated the intracellular platinum accumulation of TPT, CDDP and compound **4** using ICP-MS. A549 and HT29 cells were adhered to a multiwell plate surface and exposed to CDDP, TPT and compound **4** at a concentration of 10 μM. Pt uptake was measured following a 4 h treatment. As expected, the cells treated with TPT showed a platinum uptake 11 fold higher than the cells treated with CDDP (Fig. 3a). However, the cells treated with compound **4** displayed a poor platinum uptake, suggesting that Traniplatin-enhanced internalization could not be explained exclusively by its increased lipophilicity. We hypothesized that an active transport that relies on ATP-powered pumps[21] may be involved. In order to reduce the influence of ATP-pumps in the cancer cells, we evaluated the platinum uptake of TPT and CDDP at low (4 °C) temperature. Interestingly, the ratio of platinum internalization between TPT and CDDP dropped considerably – from 11 fold to 2 fold in A549 cells, and

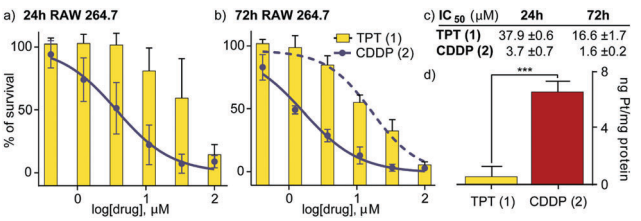

Fig. 3 Biological activity of TPT (**1**) and CDPP (**2**) on macrophages RAW 264.7. (a and b) Cytotoxicity of TPT (**1**) and CDDP (**2**) at (a) 24 h and at (b) 72 h; (c) IC_{50} was determined after 24 and 72 h treatment in macrophage RAW 264.7 cells; (d) platinum uptake of TPT (**1**) and CDDP (**2**) in macrophage RAW 264.7 cells: cells were incubated with 10 μM TPT (**1**) or CDDP (**2**) for 4 h. Platinum levels in the cancer cells were measured using ICP-MS. $^{***}p < 0.001$.

from 13 fold to 3 fold in HT29 cells – suggesting that TPT could be internalized through an active transport system (Fig. 3b). Although the underlying mechanism remains poorly understood, the correlation between signaling originating from the tumor microenvironment composed of non-malignant cells surrounding the cancer cells and the treatment efficiency suggests a key role of the stromal/immune components in modulating the patients' response to therapy.[22] Following this rationale, we evaluated the impact of TPT and CDDP on immune cells. Surprisingly, when a macrophage cell line (RAW 264.7) was treated with TPT and CDDP, an opposite biological effect was observed. Indeed, CDDP was found to be more cytotoxic than TPT after 24 and 72 hours treatment (Fig. 3a–c). Intrigued by this result, we next evaluated the platinum internalization of TPT and CDDP in macrophages RAW 264.7. Cells were treated with TPT and CDDP at a concentration of 10 μM. The platinum content was determined following a 4 h treatment. Cells treated with CDDP accumulated higher quantities of platinum than cells treated with TPT (Fig. 3d). These results support the hypothesis that an increased intracellular accumulation of TPT may not only be explained by lipophilicity-enhanced passive transport, and suggest that other mechanisms – ATP pumps or specific receptors – could be involved. However, further investigation will be needed in order to elucidate the mechanism of the internalization of TPT.

Several sources have reported a positive influence of the macrophages present at the tumor invasive front on the patients' prognosis in CRC.[23] Khorana *et al.* have shown that an increased survival of patients is associated with VEGF-expressing tumor-associated macrophages (TAM).[24] Lackner *et al.* reported that low numbers of TAM were associated with the worst prognoses.[25] In addition, Nagorsen and colleagues found that the survival of patients was significantly improved by increased intra-tumoral dendritic cell infiltration.[26] More generally, a meta-analysis of 32 studies including 2988 patients suggested that tumor inflammatory infiltrate was associated with a better prognosis in patients with colorectal cancer.[27] Therefore, the generation of drugs specifically targeting colorectal cancer cells and avoiding the immune infiltrate in the tumor may be of great therapeutic interest. Since TPT displays low cytotoxicity and a reduced platinum uptake in the macrophage cell line RAW 264.7, we hypothesized that a similar biological effect could be observed in patients' tumors using an *ex vivo* model.[28] We developed a human tumor explants model, which recapitulates *in vitro* the intra-tumoral heterogeneity – the cancer cells and the tumor microenvironment including inflammatory infiltrate, therefore allowing the evaluation of treatments that might affect both cancer epithelial cells and stromal cells. Briefly, tumor explants, obtained from the surgical specimens of untreated colorectal cancer patients, were cultured *ex vivo* in the presence of TPT (10 μM) or CDDP (10 μM) for 72 h. The cytotoxicity of the treatment was assessed by detection of the cleaved caspase-3 (a surrogate marker of apoptosis) immunohistochemically in the explants. Interestingly, immunohistochemical analysis revealed a prominent nuclear cleaved caspase-3 accumulation in the TPT 10 μM treated explant tumours (Fig. 4a, right panel) compared to the CDDP 10 μM treated samples (Fig. 4a, left panel). We performed

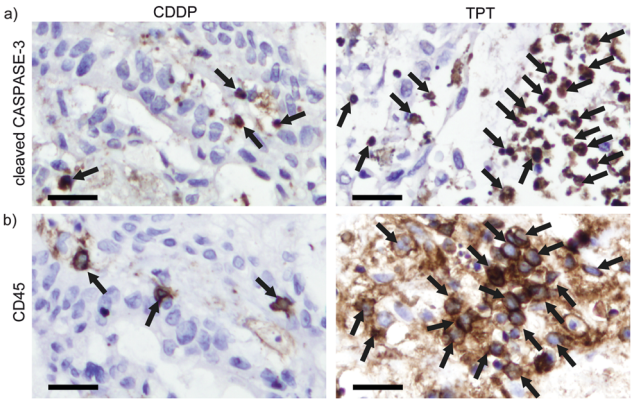

Fig. 4 TPT treated tumor explants derived from CRC patients are characterized by increased apoptosis and the presence of CD45+ immune cells. Samples were treated *in vitro* for 72 h with CDDP (10 μM) or TPT (10 μM) and compared to a non-treated control. Following *in vitro* treatment, the samples were processed for further pathological analysis – tissue fixation, inclusion and sectioning (a and b) immunohistochemical analysis of representative explants treated *ex vivo* with CDDP (left panel) or TPT (right panel). (a) Nuclear cleaved caspase-3 reactivity; arrows point to positive – apoptotic – cells in the tumor. Scale bars, 50 μm. (b) Cell membrane CD45 reactivity; arrows point to positive – immune – cells in the tumor. Scale bars, 50 μm.

CD45 immunohistochemical staining to analyse the presence of inflammatory cells under the above-mentioned conditions. A hotspot of 15 CD45+ cells was identified in a high power field in one out of four of the CDDP-treated explants.

However, we observed only a few isolated CD45+ cells in most of the CDDP 10 μM *ex vivo* treated explants (Fig. 4b, left panel). In contrast, the TPT 10 μM treated samples showed overall increased inflammation related to the tumour epithelium. In addition, hotspots of 57 and 60 CD45+ cells respectively were identified in high power fields (Fig. 4b, right panel) in two out of four of the explants. At a higher chemotherapy concentration (90 μM), only one hotspot of 5 CD45+ cells was detected in the explants treated with CDDP, while the explants treated with TPT showed the occasional presence of CD45+ cells overall and one hotspot of 10 CD45+ cells (data not shown). Overall, our data suggest that Traniplatin induces intense intra-tumoral cytotoxicity, which is associated with increased immune cell infiltrate in comparison to standard Pt-based chemotherapy (CDDP), suggesting the decreased cytotoxic activity of TPT against immune cells.

A novel Pt(IV) pro-drug, Traniplatin (TPT, **1**) based on Cisplatin (CDDP, **2**) and the marketed drug Tranilast (**3**), is reported. The newly synthesized TPT (**1**) showed enhanced cytotoxicity against colorectal and lung cancer cell lines triggering caspase-3/7 activation and apoptosis. A higher intracellular uptake of TPT was measured when compared with CDDP; however, this effect could not be exclusively associated with the increased lipophilicity of TPT. An opposite biological effect was observed in macrophages where the internalization and the cytotoxicity of TPT were significantly reduced compared to those of CDDP. A similar effect was detected in CRC patients' tumor explants treated with TPT. TPT induces intense intra-tumoral cytotoxicity, which is associated with an increased presence of intra-tumoral immune

cell infiltrate, which could be correlated with reduced cytotoxic activity against immune cells. Previous studies report a robust correlation between an improved prognosis and the presence of inflammatory cells in cancers of distinct origin including colorectal cancer. Hence, decreased chemotherapeutic cytotoxicity against CD45+ cells may improve the outcome of treated colorectal cancer patients. Therefore, TPT increased cytotoxicity against cancer cells and decreased cytotoxicity against immune cells could be of immense therapeutic interest.

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Conflicts of interest

There are no conflicts to declare.

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