Anticancer Drugs

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Immuno-Chemotherapeutic Platinum(IV) Prodrugs of Cisplatin as Multimodal Anticancer Agents**

*Daniel Yuan Qiang Wong, Charmian Hui Fang Yeo, and Wee Han Ang**

Abstract: *There is growing consensus that the clinical therapeutic efficacy of some chemotherapeutic agents depends on their off-target immune-modulating effects. Pt anticancer drugs have previously been identified to be potent immunomodulators of both the innate and the adaptive immune system. Nevertheless, there has been little development in the rational design of Pt-based chemotherapeutic agents to exploit their immune-activating capabilities. The FPR1/2 formyl peptide receptors are highly expressed in immune cells, as well as in many metastatic cancers. Herein, we report a rationally designed multimodal Pt^{IV} prodrug containing a FPR1/2-targeting peptide that combines chemotherapy with immunotherapy to achieve therapeutic synergy and demonstrate the feasibility of this approach.*

The contribution of the immune system in chemotherapy has long been discounted because cytotoxic drugs are generally believed to be immunosuppressive.[1] Consequently, new chemotherapeutic agents are often evaluated on immunodeficient mice, which neglects any possible immune contribution. However, a substantial body of recent work has challenged this assumption. There is now a growing consensus that a number of chemotherapeutics do stimulate the innate and/or the adaptive immune system and that at least part of the observed clinical therapeutic efficacy of these agents actually hinges on these off-target effects.[1] Despite this, there has been little development in the rational design of chemotherapeutic agents with the aim of combining both direct cytotoxicity and immunostimulation. Indeed, a multipronged immuno-chemotherapeutic approach would not only shrink tumors but, more importantly, would reactivate the dormant immune response against malignancies to eliminate residual cancer cells.

Pt^{II} drugs such as cisplatin, carboplatin, and oxaliplatin are effective anticancer agents in clinical use against many malignancies including testicular, ovarian, bladder, and non-small-cell lung cancer.[2] Although the formation of covalent Pt–DNA adducts is generally accepted as the principal mode of action,[3] these drugs have also been known to exert wide-ranging off-target effects on the innate and adaptive immune system.[4] Pt agents can indirectly promote immune-mediated killing of cancer cells by 1) triggering an immunogenic mode of tumor cell death through exposure of specific "eat-me" signals,[5] 2) increasing tumor cell susceptibility for T-cell killing,[6] and 3) down-regulating the immunosuppressive PD-L2 protein in a STAT6-dependent manner on tumor cells.[7] In addition, Pt agents can also directly engage immune effector functions by 1) stimulating both monocyte and natural killer (NK) cell mediated cytotoxicity,[8] 2) promoting the antigen-presenting capacity of dendritic cells,[9] and 3) reversing immunosuppressive tumor microenvironments.[7,10] The immunostimulating activity of cisplatin had already been noticed in the 1970s by Rosenberg, who first discovered the antitumor properties of cisplatin.[11] Since then, there has been more compelling empirical evidence corroborating the immunomodulating capacity of Pt-based therapy with favorable chemotherapy outcomes.[5,10,12] Nonetheless, the immune-mediating activity of Pt-based agents has been neglected in the development of new therapeutics, which has focused primarily on the principle of targeting DNA within tumor cells.

In this work, we designed a novel immuno-chemotherapeutic agent by tethering a dual-purpose peptide sequence, which behaves as both a FPR1/2-targeting moiety and an immune adjuvant, to a Pt^{IV} prodrug scaffold by using a chemoselective imine ligation strategy.[13] Pt^{IV} complexes are native prodrugs, which are pharmacologically inactive and must undergo reductive elimination by endogenous reductants to release cisplatin with concomitant dissociation of the axial ligands.[14] Formyl peptide receptors (FPRs), a family of G-protein-coupled receptors that includes FPR1 and FPR2, are selectively overexpressed in certain malignant tumors and have been implicated in mediating metastasis.[15] At the same time, FPR1/2-binding ligands, a large family that includes annexin-1, WKYMVm (m: D-Met), and fMLFK (f: formyl), are also potent immunostimulators. They activate innate effectors such as monocytes, dendritic cells, and NK cells, which lead to increased phagocytosis, chemotaxis, cytokine production, and superoxide generation.[16] FPR1/2 ligands can thus act as both targeting moieties to deliver cisplatin to FPR1/2-overexpressing cancer cells and immune cells[17] and as a potent immune adjuvant to provoke an immune anticancer response (Figure 1). Therapeutic activation of macrophages has shown promise in cancer treatment.[18] On this basis, we report a multimodal Pt^{IV} agent combining chemotherapy with immunotherapy to achieve therapeutic synergy.

We reasoned that a cisplatin-prodrug scaffold tethered to a FPR1/2-targeting peptide could simultaneously exert direct

[*] D. Y. Q. Wong,[+] C. H. F. Yeo,[+] Prof. W. H. Ang
Department of Chemistry, National University of Singapore
3 Science Drive 3, Singapore (Singapore)
E-mail: chmawh@nus.edu.sg
[+] These authors contributed equally to this work.

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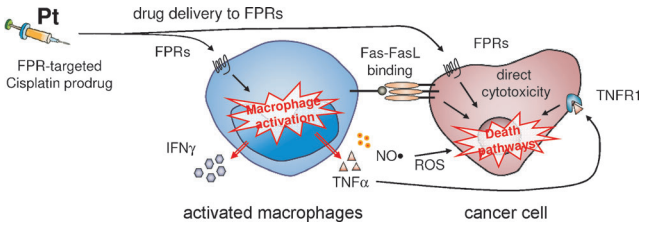
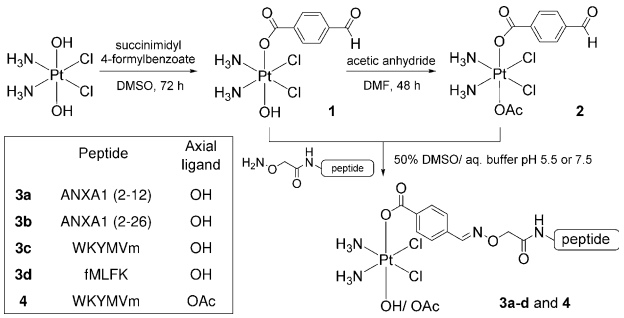


Figure 1. Putative multimodal immuno-chemotherapeutic action. FPR: formyl peptide receptor; IFN-γ: interferon γ; NO: nitric oxide; ROS: reactive oxygen species; TNF-α: tumor necrosis factor α; TNFR1: tumor necrosis factor receptor 1.

cytotoxicity against metastatic cancers overexpressing FPR1/2 receptors and activate both FPR1/2-expressing monocytes and NK cells. The Pt^{IV} scaffold was essential to accommodate the targeting peptide without modification of the cisplatin pharmacophore. The asymmetrical monofunctionalized Pt^{IV} scaffold **1** was synthesized through a *N*-hydroxysuccinimide (NHS) ester activated carboxylic acid route, which gave the monocarboxylated product preferentially over the dicarboxylated product (Scheme 1). Presumably, the NHS-activated



	Peptide	Axial ligand
3a	ANXA1 (2-12)	OH
3b	ANXA1 (2-26)	OH
3c	WKYMVm	OH
3d	fMLFK	OH
4	WKYMVm	OAc

Scheme 1. Synthesis of the Pt^{IV}–peptide conjugates.

acid was insufficiently reactive to react with the second hydroxido group, which is less nucleophilic than the first.[19] Scaffold **2** was synthesized by subsequent acetylation of **1** with acetic anhydride.

Pt^{IV}–peptide conjugates were synthesized through a previously reported chemoselective oxime ligation strategy.[13] Four different peptide agonists of FPR1/2 were conjugated to the Pt^{IV} scaffold: Annexin1 2–12 (ANXA1 2–12), Annexin1 2–26 (ANXA1 2–26), WKYMVm, and fMLFK (Scheme 1).[16c,20] Treatment of a slight excess of **1** or **2** with the desired aminooxy-functionalized peptide in DMSO/aq. buffer (pH 5.5 or 7.5) yielded the Pt^{IV}–peptide conjugates, **3a–d** and **4**, which were isolated by semipreparative HPLC and characterized by ESI-MS (see the Supporting Information).

We first investigated the ability of Pt^{IV} scaffolds **1** and **2** to generate cisplatin upon reduction because only the innate immune-modulating function of cisplatin, not any other Pt analogue, has been thoroughly studied. The prevailing hypothesis, supported by extensive studies, holds that Pt^{IV} complexes liberate cisplatin upon reductive elimination.[21] However, it has also been suggested that reduction of some *cis*-Pt^{IV} complexes can lead to the loss of any combination of the axial carboxylate and equatorial chloride ligands to yield a permutation of possible reduction products,[22] which would be undesirable because our strategy hinged upon the release of cisplatin. Therefore, we employed a combination of hydrophilic interaction chromatography (HILIC) and reversed-phase liquid chromatography (RPLC) to probe the reduction of **1** and **2** with ascorbic acid as a model of an outer-sphere reductant. HILIC and RPLC possess orthogonal retention for analytes; cisplatin has no retention on RPLC but is well resolved on HILIC.

HPLC monitoring indicated that **1** and **2** released cisplatin upon reduction. With HILIC, the signal for **1** decreased in tandem with the appearance of two emergent peaks, corresponding to the axial release of 4-carboxybenzaldehyde (4.2 min) and cisplatin (12.3 min). With RPLC, the peak for **1** (11.6 min) also decreased with the release of 4-carboxybenzaldehyde (5.0 min; Figure 2a and b). Taken together,

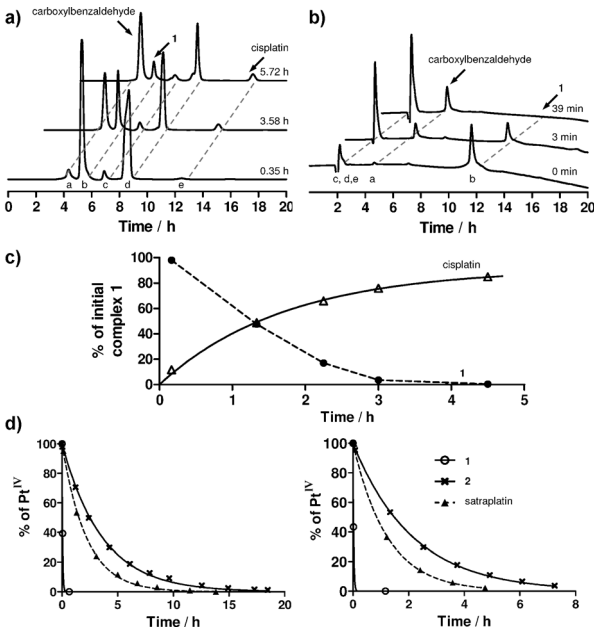


Figure 2. a) HILIC (230 nm) and b) RPLC (214 nm) chromatograms, respectively, of the reduction of **1** by ascorbic acid. **a**: 4-carboxybenzaldehyde; **b**: **1**; **c**: dehydroascorbic acid; **d**: ascorbic acid; **e**: cisplatin. c) Release profile of cisplatin over time. d) Relative rate of reduction of **1**, **2**, and satraplatin in 2 mM (left) and 4 mM (right) ascorbic acid.

these results supported the hypothesis that the reduction of **1** occurred primarily through axial dissociation because no other Pt^{II} by-products were observed. The reduction outcome of **2** was similar to that of **1** (Figure S1 in the Supporting Information). The conversion yields of **1** and **2** to cisplatin were calculated to be (89.5 ± 2.0) % (standard error (SE)) and (92.6 ± 10.1) % (SE), respectively, based on peak area against a calibration curve with HILIC monitoring (Figure 2c), which indicated that **1** and **2** are indeed true prodrugs of cisplatin. We also compared the relative rate of reduction between **1** and **2** by using RPLC (Figure 2d; rate constants in Figure S2). Intriguingly, a simple acetylation of the axial ligand led to a 77-fold decrease in the reduction rate, which

was consistent with related work by Gibson, Hambley, and coworkers suggesting that hydroxido ligands are much better bridging groups to facilitate electron transfer than acetyl carboxylates.[23]

The effects of FPR-targeting Pt^{IV}–peptide conjugates **3a**–**d** and **4** on in vitro proliferation against three FPR1/2-overexpressing tumor cell lines were assessed by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 72 h drug incubation (Figure 3). U-87MG

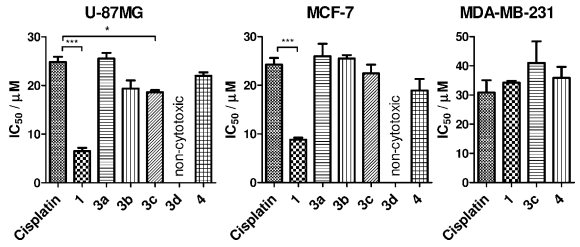


Figure 3. Absolute IC_{50} values for FPR1/2-targeted Pt^{IV}–peptide conjugates against FPR1/2-overexpressing cell lines after 72 h of incubation. Concentrations were calibrated by inductively coupled plasma–optical emission spectrometry (ICP-OES). Values are given as the mean ± the standard error of the mean (s.e.m; *: $p < 0.05$; ***: $p < 0.0001$; Student's t test).

is a highly malignant human glioblastoma cancer, the invasive behavior of which has been linked to FPR1 overexpression.[15] The two human breast cancer lines, MCF-7 and MDA-MB-231, overexpress both FPR1 and FPR2 receptors.[24] In general, all conjugates, with the exception of **3d** (the fMLFK conjugate), exhibited a comparable, if not slightly better, dose-dependent response relative to that of cisplatin. The half-maximal inhibitory concentration (IC_{50}) values followed a general trend: **1** ⋘ **3c** (WKYMVm) < cisplatin ≈ **3b** (ANXA1 2–26) ≈ **3a** (ANXA1 2–12) ⋘ **3d** (fMLFK). Against U-87MG and MCF-7, **1** was the most cytotoxic compound, followed by **3c**, which was marginally more efficacious than cisplatin, **3b**, and **3a**. Complex **3d** was effectively noncytotoxic ($IC_{50} > 100$ μM). Against MDA-MB-231, **1**, cisplatin, and **3c** all bear similar IC_{50} values. The WKYMVm conjugate **3c** was the most potent of the peptide series, so we further evaluated the cytoxicity of the related WKYMVm conjugate **4**. The IC_{50} values of **3c** and **4** were similar across all three cell lines, which indicated that the differences in reduction rate between the parent scaffolds did not affect the observed cytotoxicity in vitro. The precursor scaffold **1** was the most potent against MCF-7 and U-87MG but was comparable to cisplatin on the p53-mutant MDA-MB-231. The cytotoxicity of **1** was mostly likely attributable to nonspecific uptake by passive diffusion rather than by active targeting. In contrast to the situation in MCF-7 and U-87MG, increased accumulation of Pt was not likely to improve cytotoxicity on the cisplatin-resistant MDA-MB-231, due to its impaired p53 status.[25]

The specificity of uptake of the Pt^{IV}–WKYMVm conjugate **4** was further investigated by pretreating U-87MG cells with increasing concentrations of free competitive WKYMVm peptide in order to presaturate the FPR1/2 receptors. Thereafter, the cells were exposed to **4** for 4 h and harvested for Pt-uptake studies by using ICP-MS. Pretreatment with a one-, two-, or fivefold excess of the free WKYMVm peptide led to a corresponding decrease in the mean Pt accumulation of **4** in the treated cells to approximately 76, 64, and 35%, respectively (Figure S3). The reduction of Pt uptake by FPR1/2 ligands was attributable to competitive binding and was consistent with a model of selective drug uptake mediated by FPR1/2 receptors.[17] Some nonspecific binding may account for the fact that complete inhibition of uptake was not observed.[17]

To date, only a handful of targeted Pt^{IV}–peptide conjugates have been reported.[26] Although these targeted prodrugs demonstrated improved selectivity, their cytotoxicity does not exceed that of the parental drug, possibly due to sequestration away into lysosomal organelles. FPR1/2-targeting peptides have been validated as vectors for selective drug delivery to immune cells and have demonstrated rapid and specific peptide-drug internalization.[27] Although Pt^{IV}–peptide conjugates tend to be less potent than their Pt^{II} congener, **3c** and **4** had comparable efficacy to cisplatin, which suggested active targeting. This is reinforced by the observation that **3d** (the fMLFK conjugate) was effectively noncytotoxic, which implied that the targeting sequence plays a crucial role. Finally, uptake was FPR1/2 specific and could be inhibited by free competitive agonists in a dose-dependent manner. Complexes **3c** and **4** were selected for further studies due to their favorable cytotoxicity profile.

To demonstrate the tumoricidal activity of drug-activated peripheral blood mononuclear cells (PBMCs), we adapted an MTT-based assay described by van de Loosdrecht et al. to evaluate the immune-cell-mediated cytotoxicity.[28] Briefly, PBMCs were pretreated with Pt compounds (10 μM) for 24 h, washed, and cocultured with preseeded tumor cells at a 10:1 ratio for a further 72 h. The MTT assay was used to determine the percentage of residual viable tumor cells postincubation. Both tumor cells and PBMCs alone were plated as control groups. The cell-mediated cytotoxicity of PBMCs incubated in suspension with the Pt compounds for 24 h was significantly enhanced against the MCF-7 and MDA-MB-231 cell lines but not against U-87MG (Figure 4a). In the absence of the Pt compounds, PBMCs incubated in medium alone exerted a basal cytotoxicity with mean cell viabilities of 70.0, 87.5, and 70.1% against MCF-7, MDA-MB-231, and U-87MG, respectively, with reference to tumor cells cultured in the absence of PBMCs. Encouragingly, the FPR-targeted Pt^{IV}–WKYMVm conjugates, **3c** and **4**, were more potent than the positive control, cisplatin. It is unclear why **4** was slightly more potent than **3c**, but we postulate that the slower rate of reduction of **4** in Rosewell Park Memorial Institute (RPMI) culture medium (containing reducing glutathione) shields it from premature reduction in the extracellular milieu before uptake. Free WKYMVm peptide alone did not exert significant cell-mediated cytotoxicity, despite reportedly being a potent activator of monocytes and NK cells.[16]

The extracellular levels of the proinflammatory cytokines TNF-α and IFN-γ were measured to assess monocyte and NK cell activation (Figure 4b). These proinflammatory cytokines are the two pivotal classical mediators of the innate immune

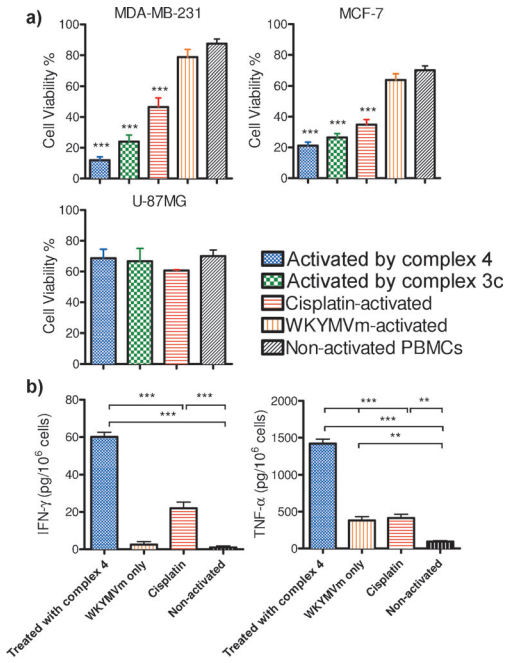


Figure 4. a) Drug-induced cell-mediated cytotoxicity. Average cell viability (%) of U-87MG, MCF-7, and MDA-MB-231 cells when coincubated with treated PBMCs after 72 h. b) Secretion of IFN-γ and TNF-α into culture medium after 24 h of treatment, as determined by a sandwich enzyme-linked immunosorbent assay. Values are given as the mean ± s.e.m. (**: $p < 0.01$; ***: $p < 0.0001$; Student's t test).

system, are themselves cytotoxic, and can also induce several possible pathways of contact-mediated killing of tumor cells downstream. Treatment of PBMCs with **4** dramatically enhanced secretion of both TNF-α and IFN-γ relative to that from nontreated PBMCs ($p < 0.0001$) and the positive control cisplatin ($p < 0.0001$). Cisplatin treatment alone significantly increased both TNF-α and IFN-γ secretion relative to that from nontreated PBMCs ($p < 0.01$ and $p < 0.0001$, respectively) but to a much lesser extent than **4**, which is consistent with the results of the cell-mediated cytotoxicity assay. Cisplatin is a potent activator of monocytes[8a,29] and NK cells,[8b,c,29] both in vitro and in vivo. Tumoricidal activity due to cisplatin activation is poorly understood but is believed to be mediated by the proinflammatory cytokines TNF-α and IFN-γ.[30] Cisplatin treatment promotes contact-dependent cell death, which is either due to enhanced NO production or to cytokine-dependent contact-mediated apoptosis of tumor cells due to monocyte membrane-bound exposure of the apoptosis-inducing ligands FasL and/or TNF-α.[31] It is not known why the treated PBMCs were ineffective against U-87MG, but this immune evasion may be due to differences in intrinsic susceptibility to activated macrophages. Intriguingly, we observed a disconnection in the susceptibility of the various cell lines to the immune-mediated cytotoxicity and the direct cytotoxicity. For instance, the p53 mutant MDA-MB-231 was resistant to DNA-damage-triggered cell death but was the most vulnerable to immune-mediated cytotoxicity. This observation supports the utility of a multipronged immuno-chemotherapy approach in the treatment of drug-resistant tumors.

Over the last decade, there has been substantial evidence supporting the pivotal role of the immune system in inducing tumor regression after conventional chemotherapy. Even though Pt-based agents dominate conventional chemotherapy and are included in around 50% of all chemotherapy combinations,[32] the immunomodulating capacity of these agents remains largely unexplored. We demonstrated herein that a rationally designed multimodal immuno-chemotherapeutic Pt-based agent capable of selective cancer cell targeting and eliciting an immune response is feasible. We believe that this could pave the way for clinically useful immuno-chemotherapeutic Pt-based agents with improved therapeutic outcomes.

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