Antitumor Agents

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The Prodrug Platin-*A*: Simultaneous Release of Cisplatin and Aspirin**

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Abstract: *Cancer-associated inflammation induces tumor progression to the metastatic stage, thus indicating that a chemo-anti-inflammatory strategy is of interest for the management of aggressive cancers. The platinum(IV) prodrug Platin-A was designed to release cisplatin and aspirin to ameliorate the nephrotoxicity and ototoxicity caused by cisplatin. Platin-A exhibited anticancer and anti-inflammatory properties which are better than a combination of cisplatin and aspirin. These findings highlight the advantages of combining anti-inflammatory treatment with chemotherapy when both the drugs are delivered in the form of a single prodrug.*

Chronic inflammation plays an important role in approximately 20% of human cancers.[1] Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer death in men in the United States.[2] Patients with PCa inevitably progress to a hormone-independent disease. PCa that progresses in the presence of an androgen blockade is defined as castration-resistant prostate cancer (CRPC).[2] A complex immune-mediated process and inflammation play crucial roles in PCa progression in the castrate setting and cancer-associated inflammation contributes to the formation of metastasis.[3] *Cis*-diamminedichloridoplatinum(II), or cisplatin,[4] is currently one of the most effective anticancer drugs available for treating a variety of solid tumors.[5] Resistance to apoptotic death is a characteristic feature of advanced PCa[6] and is one of the reasons for the failure of a cisplatin-based therapeutic strategy for hormone refractory disease.[7] Nonsteroidal anti-inflammatory drugs (NSAIDs) can be an attractive additive to chemotherapeutic approaches for PCa because of their ostensive potential in cancer chemoprevention.[8] The primary target of NSAIDs is cyclooxygenase (COX) isoforms, COX-1 and COX-2, which catalyze the rate-limiting step in the formation of prostaglandins (PGs).[9] PGs are the group of lipid molecules derived from arachidonic acid and play a key role in generating inflammatory response. The inducible isoform COX-2 and its products, especially PGE2, are involved in inflammatory responses, inhibition of apoptosis, and induction of resistance. Increased levels of PGE and COX-2 mRNA are overexpressed in 83% of human PCa samples.[10] The use of cisplatin is limited because of its side effects such as nephrotoxicity and ototoxicity. Nephrotoxicity can be reduced using saline hydration. However, there are no protective modalities for cisplatin ototoxicity.[11] Acetylsalicylic acid, or aspirin, which is known to inhibit COX-1 and COX-2 irreversibly through transesterification between acetylsalicylic acid and the Ser-530/516 residue of COX,[12] also has the potential to reduce the severity of cisplatin-induced side effects related to hearing, balance, and the kidneys.[11] Aspirin and its metabolite salicylate induce several anti-inflammatory cytokines to reduce inflammation.[13]

A combination of cisplatin and aspirin can be an attractive strategy for managing highly aggressive PCa. Major obstacles in administering free-drug formulations include the definitive exposure to the targets of interest, individual pharmacokinetics, and biodistribution parameters. These factors are extremely difficult to control when drugs are individually administered. However, construction of a single prodrug containing a drug combination can potentially overcome these challenges. Thus, an alternative way for delivering a therapeutic combination of cisplatin and aspirin is to fabricate a platinum(IV) prodrug which can be reduced to give cisplatin and aspirin. Until now, no examples of a prodrug containing both cisplatin and aspirin have been reported. With these challenges in mind, we constructed a platinum(IV) prodrug, Platin-*A*, with the ability to release cisplatin and aspirin for their respective biological actions (Scheme 1).

Platinum(IV) prodrugs exhibit greater kinetic inertness compared to the more labile cisplatin.[14] In Platin-*A*, the aspirin moiety was introduced by a reaction of *c*,*c*,*t*-[Pt-$(NH_3)_2Cl_2(OH)_2$] with aspirin anhydride (see Figures S1 and S2 in the Supporting Information). Platin-*A* was characterized using several spectroscopic and analytical techniques (see Figures S3–S5). By mimicking the narrow pH range of 7.35–7.45 of blood, it was found that the reduction potential of Platin-*A* at 7.4 pH is −536 mV versus the normal hydrogen electrode (see Figure S6). Dysregulated pH is an adaptive feature of most cancers.[15] In normal cells, the intracellular pH (pH_i) is about 7.2 which is lower than the extracellular pH

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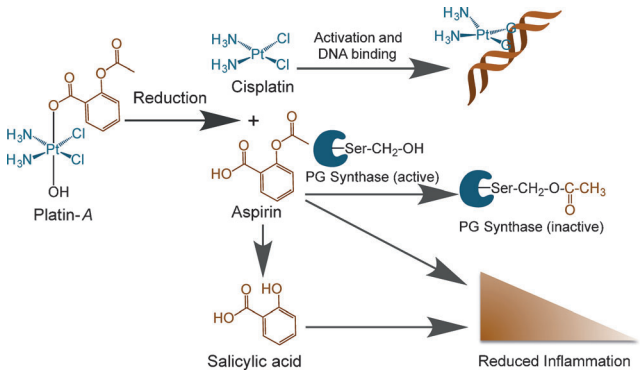
Supporting information for this article (detailed descriptions of materials and instruments, synthesis and characterizations of Platin-A, and other experimental details) is available on the WWW under http://dx.doi.org/10.1002/anie.201308899.



Scheme 1. Structure of Platin-*A* and its mechanism of action.

(pH_e) of about 7.4. In cancer cells, the pH_i value is greater than 7.4 and the pH_e value is less than 7.1. Reduction properties of Platin-*A* were studied at pH 6.4 to mimic the reduced extracellular pH_e of cancer. At pH 6.4, a positive shift of 42 mV was observed (Figure S6), and indicated that the reduced pH_e in the cancer microenvironment would facilitate reduction of Platin-*A* to release cisplatin and aspirin. The remarkable anticancer activity of cisplatin is due to its inherent proficiency to bind with the N7 position of guanine bases, thus leading to DNA damage.[4b] To mimic the intracellular reduction, Platin-*A* was reduced with sodium ascorbate in the presence of 2′-deoxyguanosine 5′-monophosphate sodium salt hydrate (5′-GMP) as a truncated version of DNA, and the products were analyzed by matrix-assisted laser desorption ionization (MALDI)/time of flight (TOF) mass spectrometry (MS). Platin-*A* reduction followed by reaction with 5′-GMP showed formation of a Pt^{II}/5′-GMP-bisadduct, $[Pt(NH_3)_2(5'\text{-GMP-N7})_2]$ (m/z 922, see Figure S7). Binding studies with 5′-GMP provided strong evidence that cisplatin released upon reduction of Platin-*A* will interact with nuclear DNA efficiently. The release of aspirin from Platin-*A* was analyzed by high-performance liquid chromatography (HPLC; see Figure S8). The HPLC chromatogram from the reduction reaction and comparison of the traces with chromatograms of pure samples of aspirin and salicylic acid indicated that Platin-*A* initially produces aspirin upon reduction. As time progresses, the formation of increasing amounts of salicylic acid, the main metabolite of aspirin, was detected (Figure S8). This observation supported the release of aspirin moiety from Platin-*A* in either its pristine form or its final metabolite form for COX inhibitory and anti-inflammatory activities, respectively.

High expression of COX-2 is found in various cancers, however, for PCa cell lines as well as tissue, contradictory results exist.[16] We therefore tested anti-proliferative properties of Platin-*A* on androgen-responsive LNCaP and androgen-unresponsive PC3 and DU145 PCa cells which differ in their malignant potentials. Platin-*A* demonstrated an IC_{50} value comparable to that of cisplatin alone and an equimolar mixture of cisplatin and aspirin in PC3 cells (see Figure 1 and Table S1). Similar trends were observed in DU145 and LNCaP cells for Platin-*A* and cisplatin (see Figure 1 and Table S1). Platin-*A* exhibited a slightly higher IC_{50} value than an equimolar mixture of cisplatin and aspirin in DU145. However, in LNCaP cells, Platin-*A* activity was better than an equimolar mixture of cisplatin and aspirin (see Figure 1 and Table S1). In general, platinum(IV) compounds are less cytotoxic compared to their active platinum(II) form. Comparable cytotoxicities of Platin-*A* and cisplatin demonstrated unique anti-proliferative potency of Platin-*A* in PCa cells. To evaluate whether Platin-*A*-dependent inhibition of cancer cell proliferation is associated with apoptosis, an AlexaFluor 488 Annexin V/propidium iodide (PI) staining

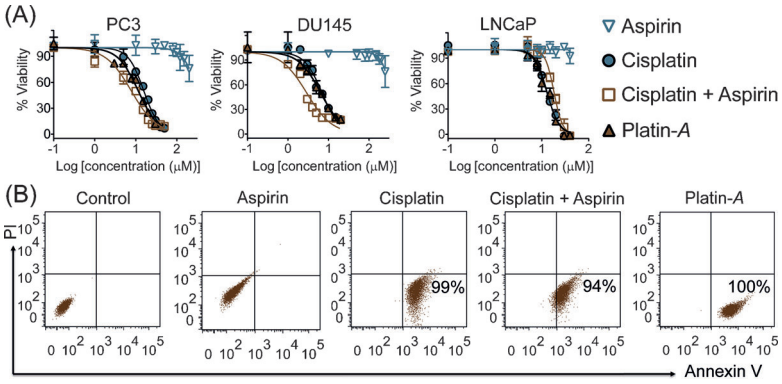


Figure 1. A) Representative cytotoxic profiles of Platin-*A*, cisplatin, and an equimolar mixture of cisplatin and aspirin in PCa cell lines. B) Apoptosis inducing property of Platin-*A* by annexin V-FITC/PI staining of PC3 cells. Four distinct phenotypes: viable cells (lower left quadrant); cells at an early stage of apoptosis (lower right quadrant); cells at a late stage of apoptosis or necrosis (upper right quadrant); debris (upper left quadrant).

assay was carried out in PC3 cells and the data were analyzed by fluorescence-assisted cell sorting. PC3 cells were incubated with aspirin (25 μM), cisplatin (25 μM), an equimolar mixture of cisplatin and aspirin (25 μM), and Platin-*A* (25 μM) for 6 hours. Additionally, cells treated with etoposide (100 μM, 12 h) and H_2O_2 (1 mM, 45 min) were used as positive controls of apoptosis and necrosis, respectively (see Figure S9). A high level of apoptosis was induced by Platin-*A*. The apoptosis-inducing property of Platin-*A* was very similar to that of cisplatin and an equimolar mixture of cisplatin and aspirin. Aspirin alone did not show any changes in healthy PC3 populations under these conditions (Figure 1B).

In vitro COX inhibitory properties of Platin-*A* were studied using an enzyme immunoassay (EIA). Platin-*A* showed very similar inhibition of both COX-1 and COX-2 as shown by aspirin in a concentration-independent manner (Figure 2; see Figure S10). Aspirin is known to be more potent than salicylate as an inhibitor of COX-1 or COX-2.[17] Comparable COX-1 and COX-2 inhibitory properties of Platin-*A* indicated that reduction of Platin-*A* first releases aspirin as supported by our HPLC study (Figure S8). These remarkable COX inhibitory properties of Platin-*A* indicated its potential in reducing tumor-associated inflammation.

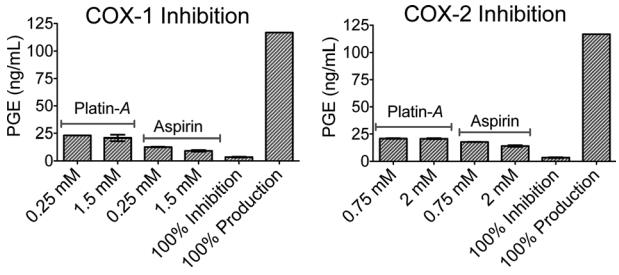


Figure 2. Inhibition of ovine COX-1 and COX-2 by Platin-*A*.

Many cancers show elevated levels of COX-2, however, COX-2 contributions to PCa and its regulation by inflammatory cytokines are controversial. Basal COX-2 levels were very low in PC3 cells and no effect was observed in the presence of all the three test articles (see Figure 3 A and Figure S11). We, therefore investigated enhancement of COX-2 levels in response to COX-2-inducing cytokine tumor necrosis factor-α (TNF-α) for its strong COX-2 gene expression inducing property by nuclear-factor-κB (NF-κB)-binding motifs in the COX-2 promoter[18] (see Figure 3 B and Figure S11). Treatment of PC3 cells with TNF-α (20 ng mL^{-1}) for 2 hours followed by the examination of the COX-2 expression pattern upon treatment with Platin-*A* (1 μM), cisplatin and aspirin (1 μM), and aspirin (1 μM) for 2 hours was studied. An elevated COX-2 expression was observed in TNF-α-stimulated PC3 cells and the levels were reduced upon treatment with Platin-*A* (see Figure 3 B and Figure S11). An equimolar mixture of cisplatin and aspirin showed a less prominent effect on these activated cells, and the level of inhibition shown by aspirin was similar to that observed with Platin-*A* (see Figure 3 and Figure S11). Significant inhibition of cellular COX-2 by Platin-*A* further supported its unique ability to reduce tumor-associated inflammation.

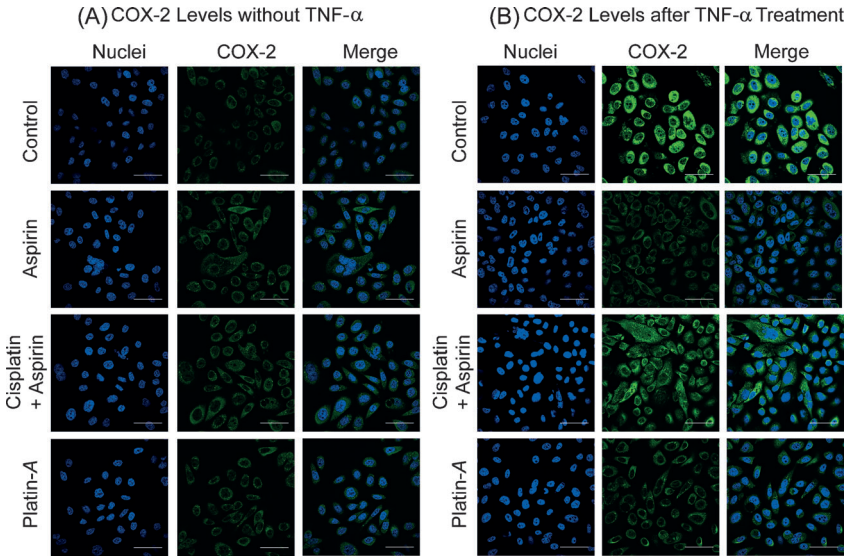


Figure 3. Immunofluorescence analysis of COX-2 expressions in PC3 cells. A) COX-2 expressions in PC3 cells and the effect of aspirin (1 μM), and an equimolar ratio of cisplatin and aspirin (1 μM each), and Platin-A (1 μM). B) effect on the COX-2 levels in PC3 cells upon treatment with TNF-α, and the effect of aspirin (1 μM), and an equimolar ratio of cisplatin and aspirin (1 μM each), and Platin-*A* (1 μM) on TNF-α stimulated cells. Cells were DAPI-stained in blue. COX-2 were labeled with a primary rabbit polyclonal antibody and subsequently labeled with a secondary AlexaFluor 488 goat anti-rabbit antibody. All scale bars are 25 μm.

Pro-inflammatory cytokines, TNF-α, and interleukin (IL)-6, are important for proliferation, survival, metastasis, and escape from immune surveillance of cancers.[19] Chronic inflammation by activation of toll-like receptors (TLRs) on cancer cells creates a tumor microenvironment which impairs the antitumor function of the immune system.[20] The cytokines TNF-α and IL-6 are potent activators of NF-κB, a key modulator of inflammation-induced carcinogenesis. Enhanced TNF-α secretion mediates cisplatin nephrotoxicity.[21] The anti-inflammatory cytokine IL-10 inhibits inflammatory and cytotoxic pathways in cisplatin-induced acute renal injury.[22] TLR-mediated pro-inflammatory cytokine production from tumor-associated macrophages (TAMs) play a key role in tumor progression and cancer cell metastasis.[23] We therefore investigated effects of Platin-*A* on lipopolysaccharide (LPS), an exogenous ligand for TLR4-activated RAW 264.7 macrophages, to mimic the inflammatory environment in cancer.[20] First, we evaluated the effects of Platin-*A*, cisplatin, a combination of cisplatin and aspirin, and aspirin alone on pro-inflammatory cytokines IL-6, TNF-α, and anti-inflammatory IL-10 production in LPS-stimulated RAW 264.7 macrophages using an enzyme-linked immunosorbent assay (ELISA).[24] Stimulation of RAW cells with LPS markedly increased IL-6 and TNF-α production, compared with that generated under the control conditions (Figure 4 A). Under our experimental conditions, we did not observe any secretion of IL-10 from RAW macrophages upon stimulation with LPS. Treatment of LPS-activated macrophages with 10 μM of Platin-*A* for 12 hours significantly reduced the levels of IL-6 and TNF-α. Cisplatin, under the same conditions, did not show any effect on the levels of these cytokines (Figure 4 A). To exclude the possibility that the decrease in the cytokines levels was simply due to the cytotoxicity of Platin-*A*, cell viability was evaluated. Platin-*A* (10 μM) did not affect cell viability when incubated for 24 h (see Figure S12).

Secretion of anti-inflammatory cytokine IL-10 by LPS-activated macrophages in the presence of Platin-*A* indicated its unique anti-inflammatory properties. Cisplatin, aspirin, or an equimolar mixture of cisplatin and aspirin did not show any secretion of IL-10.

We next assessed the effect of pretreatment of Platin-*A* on macrophages prior to LPS stimulation. As shown in Figure 4 B, stimulation of RAW macrophages with LPS led to

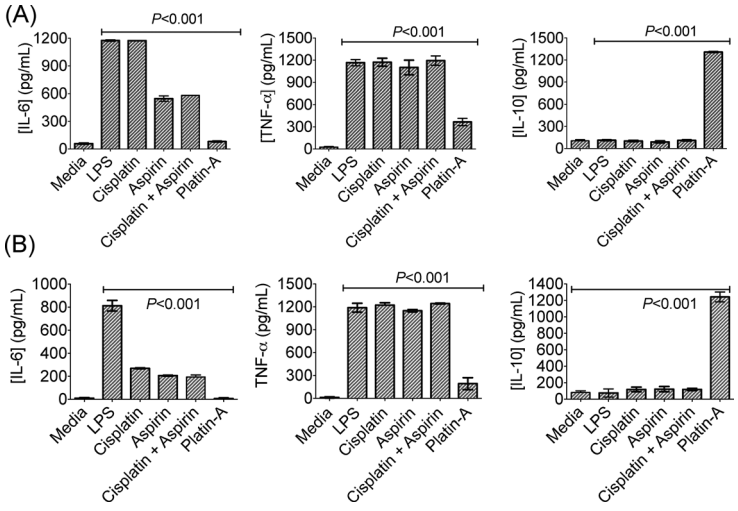


Figure 4. Anti-inflammatory properties of Platin-*A*. A) RAW 264.7 cells were treated with LPS and the effects of cisplatin, aspirin, an equimolar mixture of cisplatin and aspirin, and Platin-*A* on the levels of IL-6, TNF-α, and IL-10 were studied using ELISA. B) RAW 264.7 macrophages were treated with cisplatin, aspirin, an equimolar mixture of cisplatin and aspirin, and Platin-*A* followed by addition of LPS and the levels of IL-6, TNF-α, and IL-10 were studied using ELISA. Statistical analyses were performed by using one-way ANOVA with Tukey post-hoc test.

a significant increase in the levels of IL-6 and TNF-α in the cell-conditioned media after 12 hours. Pretreatment of these macrophages with Platin-*A* significantly inhibited LPS-induced IL-6 and TNF-α production at a low concentration of 10 μM. A combination of pretreatment with Platin-*A* and LPS showed an enhanced level of IL-10 (Figure 4B). These results together indicated that Platin-*A* is efficient in curtailing inflammatory response. Pretreatment of macrophages with cisplatin, aspirin, and an equimolar mixture of cisplatin and aspirin combination did not show any preventive action against LPS stimulation.

Based on our data, we sketched out the possible mechanism of action of Platin-*A* in Figure 5. Platin-*A* is expected to undergo reduction in the reducing tumor microenvironment to release the active drugs, cisplatin and aspirin. The active form of platinum(II) acts on the nuclear DNA. Aspirin inhibits COX enzyme and along with its metabolite salicylate controls the levels of inflammatory responses in TAMs.

In conclusion, the unique chemo-anti-inflammatory molecule Platin-*A*, a prodrug for cisplatin and aspirin, was synthesized and characterized. A favorable reduction pattern of Platin-*A* was observed with the release of the biologically active platinum(II) form with concurrent liberation of aspirin. Platin-*A* showed cytotoxicity profiles comparable to cisplatin, and demonstrated unique apoptosis inducing potency in PCa cells. Owing to its distinctive formulation bearing aspirin, Platin-*A* showed anti-inflammatory effects and a unique ability to inhibit intracellular COX-2. These results encourage further development of Platin-*A* as a potential candidate for cancers characterized with chronic inflammations. This work highlights the opportunities to uniquely combine NSAIDs, such as aspirin, with a cisplatin treatment regimen in the form of a single prodrug to increase efficiency and reduce side effects such as ototoxicity of chemotherapy.

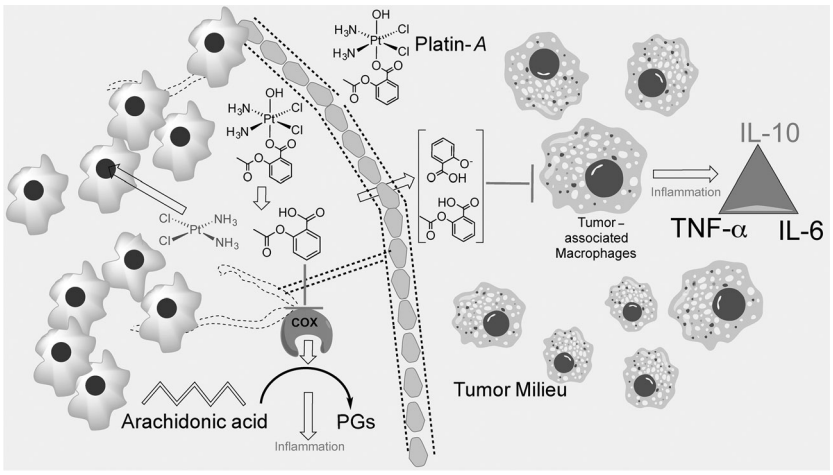


Figure 5. Possible mechanism of action of Platin-*A*.

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