Novel aspects of the preclinical pharmacology of platinum compounds

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Abstract
Platinum compounds are widely used antitumor agents known to interfere with DNA function by forming DNA crosslinks and DNA-protein crosslinks. Because of their electrophilicity, platinum compounds can interact with nucleophilic residues of all macromolecules. Consequently, this cross-linking inhibits DNA replication in cancer cells. Immunogenic and immunomodulating effects have been ascribed to platinum drugs, with differences and similarities among cisplatin, carboplatin and oxaliplatin. On the one hand, cisplatin is generally unable to induce immunogenic cell death; on the other hand, oxaliplatin appears to be a good inducer, thanks to its capability to efficiently trigger calreticulin exposure to the tumor cell plasma membrane. Conversely, cisplatin, carboplatin and oxaliplatin can relieve immunosuppressive networks e.g., by decreasing PDL-1 and PDL-2 in dendritic and tumor cells. Such drugs are also capable of modulating MHC molecules via IFN-β production and T-cell mediated lysis. The concentrations appear to be key in determining the immunomodulatory properties of these cytotoxic agents, with low in vivo doses usually playing stimulatory effects. As predicted from preclinical models, supportive results have emerged from clinical studies, particularly those based on chemotherapeutic regimens of platinum compounds combined with immunotherapeutics. Future therapeutic interventions are expected to benefit from a better definition of the molecular effects of platinum compounds on the immune system.

Keywords
Cisplatin; Carboplatin; Oxaliplatin; Immunogenic cell death; Immunostimulation

1. Introduction
Platinum (Pt) compounds are a small class of conventional antitumor agents employed in the treatment of a variety of tumors, including common and rare neoplastic diseases. The first discovered Pt agent is cisplatin [cis-diammine (dichloro) platinum (II)] which was approved in 1978 for the treatment of metastatic testicular cancer, ovarian and bladder cancer [1, 2] (Fig. 1). In more recent years, cisplatin has been employed in the treatment of additional malignancies, after the identification of the molecular features favoring its activity. For instance, the drug is used in the treatment of triple negative breast cancer [3, 4] and prostate cancer [5] [ClinicalTrials.gov Identifier: NCT03275857].

Carboplatin [cis-diammine(cyclobutane-1,1-dicarboxylato) platinum(II)] and oxaliplatin cis-[oxalato (trans-l,2-diamino-cyclohexane) platinum(II)] were approved in 1989 and 2002, respectively [6, 8]. Other Pt compounds are in clinical use in non-Western countries (nedaplatin, lobaplatin, heptaplatin) [9]. Additional Pt agents have undergone clinical evaluation (e.g., satraplatin), but have failed to achieve approval [10].

Pt compounds are known to interfere with the normal functions of DNA as a consequence of the generation of crosslinks in the same DNA strand (intrastrand crosslink), in opposite strands (interstrand crosslinks) or between proteins and DNA. Carboplatin, which behaves as a pro-drug because it is less reactive than cisplatin and its leaving groups are released slowly, forms DNA adducts that are identical to those formed by cisplatin [6]. Its spectrum of activity is superimposable to that of cisplatin, whereas oxaliplatin - which generates DNA adducts that, differently from cisplatin, are not recognized by the DNA mismatch repair (MMR) system - is mainly used for the treatment of colon carcinoma [6]. When hydrolyzed in biological fluids, Pt compounds behave as electrophilic species which potentially interact with all nucleophilic residues of biomacromolecules. Indeed, effects which are not related to their interaction with DNA are very likely for these drugs, such as alterations in the plasma membrane, the endolysosomal compartments and mitochondria [11]. Such effects may contribute to drug resistance which represents a major hindrance towards the cure of cancer. In addition to factors preventing the interaction of the drug with its cellular target (e.g. efflux transporters) [12], resistance to drug-induced cell death, self-sufficiency in growth and survival signals, have been shown to contribute to drug resistance [13]. Besides, the microenvironment promotes chemoresistance through changes in the extracellular matrix, hypervascularization, hypoxia and paracrine factors [14, 15]. In this context, extracellular vesicles may sustain evasion from cell death and confer chemoresistance [16].

Here, we review both early and novel insights regarding the immunogenic and immunomodulating effects of Pt compounds. Indeed, a better understanding of the interference of Pt drugs with components of the immune system may be helpful to enhance the efficacy
of Pt-based chemotherapeutic regimens including those combined with agents targeting immune checkpoints.

2. Immunogenic cell death

Immunogenic cell death (ICD) is a well-characterized process that occurs when dying cells - uninfected but expressing a specific tumor antigen - trigger a protective immune response [17, 18]. This type of cell death has been shown to be induced by conventional chemotherapy, particularly by anthracyclines and by oxaliplatin but not by cisplatin [19, 20] (Fig. 2). For a drug to induce cell death, specific events are needed, the most critical of which is the translocation of calreticulin from the lumen of the endoplasmic reticulum (ER) to the plasma membrane [20]. The translocation of the pre-formed protein occurs much earlier than other events characterizing apoptosis, like phosphorylase exposure on the cell surface or nuclear fragmentation. Calreticulin acts as “eat-me” signal allowing phagocytosis of dying tumor cells by dendritic cells (DCs), essential for adaptive immune responses, given their capacity to cross-present exogenous antigens to T lymphocytes. Phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2α), which blocks protein synthesis and allows cells to adapt to ER stress has been demonstrated to be critical for the induction of ICD, although this post-translational modification is not sufficient for calreticulin exposure [20] (Obeid 2007). The reason for the inability of cisplatin to induce ICD has been addressed in preclinical studies – mostly carried out with rather high drug concentrations and long exposure times, i.e. 150 μM for 16 h - which have shown that cisplatin is unable to phosphorylate eIF2 alpha [18]. However, if cisplatin is combined with agents inducing ER stress (e.g., tunicamycin) it becomes capable to induce ICD. After calreticulin and heat shock protein exposure, tumor antigen uptake occurs, the High Mobility Group Box 1 (HMGB1) protein is released by tumor cells, thereby stimulating Toll-Like Receptor 4 (TLR-4) on Antigen Presenting Cells (APCs), i.e. DCs. ATP released from dying cells stimulates the purinergic P2X receptors (P2XR) on DC which in turn produce IL-18 [21].

The lack of immunogenicity of cisplatin-induced cell death has prompted researchers to search for approaches rendering cisplatin capable of inducing cell death with immunogenic features (Fig. 2). Specifically, using a murine cell line engineered to express calreticulin on the surface of cancer cells, a stimulation of the dying tumor cell capability to trigger an immune response to tumor re-challenge was observed after cisplatin treatment (150 μM, 24 h exposure), showing that a genetic manipulation of ER stress response can enhance cisplatin efficacy [22]. Recently, the up-regulation of calreticulin achieved in malignant pleural mesothelioma cells by over-expression of C/EBP-β LIP, a transcription factor activated in response to ER stress, was shown to trigger tumor cell phagocytosis by DCs and to expand CD8+ CD107+ cytotoxic T lymphocyte (CTL)-mediated antitumor response with the activation of apoptosis via CHOP/TRB3/caspase 3 [23]. Besides, in drug-resistant malignant cell lines of distinct tumor lineages characterized by p53 defects, Zn++ supplementation was shown to reactivate dysfunctional p53 and to enhance cell sensitivity to cisplatin [24]. Notably, in the presence of ZnCl2, dying cells could activate the immune system by promoting calreticulin exposure, as shown by induction of DCs maturation by tumor cells pre-incubated with 13.3 μM cisplatin for 16 h, followed by co-culturing for 24 h with immature DCs [24]. This drug concentration – upon 24 exposure - displayed the ability to kill 20% of cells by itself and around 40% of cells in cells pre-treated with ZnCl2, thereby suggesting a potentiation of cisplatin effect. This study further supports that the induction of calreticulin translocation to the cell surface by Zn++ supplementation is required for the immunogenicity of cisplatin-triggered cell death and shows that low micromolar concentrations of cisplatin under long-term drug exposure can result in ICD [24]. Moreover, the vitamin B precursor pyridoxine was found to display a synergistic effect with cisplatin used at a suboptimal dose (1.5 mg/Kg) in experiments carried out in immunocompetent mice, but not in nude mice [25]. The efficacy of the combined treatment was ascribed to the increased induction of eIF2α phosphorylation and calreticulin exposure observed in cells exposed to the two compounds as compared to singly-treated cells. Because in this study some signs of ICD were evident also in cells treated with cisplatin alone, it seems reasonable that the molecular background of the specific tumor as well as the concentrations and exposure times of cisplatin used for treatment (1-40 μM for 48 h), contribute to determine if the compound per se can induce ICD. In fact, it has been previously reported that cisplatin is capable to induce ER stress [26], a feature that may result in eliciting an immune response [23].

Treatment with cisplatin in an in vivo preclinical model has been shown to result in the release of damage-associated molecular patterns (DAMPs) other than calreticulin, including heat shock proteins and HMGB1 [27], in spite of the inability of the drug to induce ICD. Approaches which interfere with ER, specifically ER stressors can reverse such an inability, as shown for instance with electroporation. Indeed, based on the promising features of electrochemotherapy, (i.e., the delivery of chemotherapeutic drugs by electroporation) with intratumoral cisplatin [28, 29], in a recent study Ursik and colleagues compared the effects of cisplatin and oxaliplatin upon electrochemotherapy using a murine melanoma [30]. They found that this mode of delivery allowed to efficiently accumulate Pt drugs in malignant cells and exerted similar DNA platination with cisplatin and oxaliplatin, when a higher concentration of oxaliplatin was used. This approach facilitating the accumulation of both cisplatin and oxaliplatin, resulted in the activation of ICD, a phenomenon that, again, highlights the possibility to reverse drug incapability to activate ICD [30].

The available evidence supports that there are conditions that favor the formation of neo-antigens and may therefore facilitate the occurrence of ICD [31]. In this regard, it is important to recognize that one of the key characteristics of oxaliplatin is its ability to surmount resistance to cisplatin-mediated by DNA MMR defects [32, 33]. Several preclinical studies have shown that cisplatin-resistant cells do not express or down-regulate components of the DNA MMR system such as MLH1 or MSH2, features that have been associated with microsatellite instability (MSI) [33, 34]. MSI, frequently observed in colorectal carcinoma, consists of a difference in the number of repeats between normal and tumor tissue or between sensitive and chemoresistant cells. In cells with DNA MMR defect, mis-incorporation, insertions and deletions introduced by DNA polymerase slippage (frequent in the presence of repeats) are not recognized and corrected, with the generation of length variation accumulation at microsatellites, short non-coding sequences also known as short tandem repeats (STR). MSI which occurs also in genomic coding regions can alter the reading frame and lead to the translation of peptides with altered amino acid sequences at the C terminus. Such peptides represent neo-antigens and are very immunogenic. In fact, MMR deficiency has been shown to predict response of solid
tumors to programmed cell death 1 (PD1) protein inhibition [31].

3. Immunomodulating effects of platinum compounds

The available evidence supports that chemotherapy is endowed with immunomodulating properties that are distinct from the ability to induce ICD. Overall, the immunostimulatory activity of Pt compounds has been linked to their ability to a) down-regulate the immunosuppressive microenvironment, b) enhance the expression of MHC class I molecules, c) promote recruitment and proliferation of immune effector cells, and d) increase the lytic activity of CTLs [35, 36] (Fig. 3).

3.1. Modulation of immunosuppression

A stimulation of immune responses against cancer by Pt drugs has been recently reported in humans and mice by Lesterhuis and colleagues, who also provided insights on immunosuppression mechanisms [37]. In their study, cisplatin, but also carboplatin and oxaliplatin, have been shown to markedly decrease the expression of the immunesuppressive molecule programmed death receptor-ligand 2 (PDL-2), and to a reduced extent of programmed death receptor-ligand 1 (PDL-1) when used at concentrations comparable to those employed in the clinics; the effect was observed both in DCs and in tumor cells [37]. In particular, treatment of monocyte-derived DCs with oxaliplatin, carboplatin or cisplatin during 48 h of cytokine maturation resulted in enhanced allogenic T cell stimulatory capability. Besides cytokine-induced maturation, also TLR-induced stimulatory potential was increased by Pt compounds; of note, the up-modulation of T-cell proliferation was concentration-dependent and was achievable both during or after DC maturation, therefore resulting independent of DC activation or maturation. In this setting, the increased immunostimulatory potential of DCs upon Pt drug exposure was not associated with up-regulation of class I and II MHC molecules, of co-stimulatory CD80 and CD86, nor of a consistent pattern of modulation of pro- or anti-inflammatory cytokines (e.g., TNF α, IL-8) [37]. Functional approaches demonstrated that enhanced immunogenicity of Pt-treated DCs was due to Signal Transducer and Activator of Transcription 6 (STAT6) which is critical for regulation of PDL2; in fact, exposure to Pt compounds produced a decrease in Stat6 phosphorylation [37]. Functional experiments using melanoma cells exposed to a cytokine cocktail inducing PDLs indicated that cisplatin decreased STAT6 phosphorylation in parallel with PDL1 and PDL2 levels also in tumor cells. It is unclear if oxaliplatin induced a similar effect, but it is likely that with appropriate concentrations, this phenomenon occurs with oxaliplatin and carboplatin. Interestingly, antigen-specific T cells displayed increased capacity to recognize cisplatin-treated cells [37]. The concentrations of Pt drugs used in this study were in the micromolar range for oxaliplatin and cisplatin, whereas higher concentrations were used for carboplatin, owing to its pro-drug nature. These concentrations are likely to be clinically relevant although a rather long-term exposure (48 h) was necessary to obtain modulatory effects.

Fig. 1. Structures of the most commonly used platinum compounds. The dates of approval for clinical use are indicated together with the mechanism of action and cell response.
3.2. Modulation of MHC molecules

Several studies have reported the ability of Pt compounds to up-modulate MHC molecules. In a report focusing on the immunomodulating effects of chemotherapy in breast cancer cells [38], cisplatin has been shown to increase the expression of MHC class I through IFN-β signaling. The mechanism underlying this phenomenon has been well characterized for the DNA topoisomerase I inhibitor topotecan for which drug-induced IFN-β and MHC I expression require active DNA synthesis and depend on NF-κB, which is known to transcriptionally induce genes encoding for several cytokines including IFN-β, whose promoter contains a NF-κB binding site [38]. The implication of IFN-β in MHC I modulation is supported by the evidence that a neutralizing antibody and silencing of the type I interferon receptor subunit 1 (IFNAR1) result in decreased drug-induced MHC I expression [38]. Thus, a paracrine/autocrine production of IFN-β seems to be key for MHC I induction. The mechanism might be shared by different chemotherapeutic agents including cisplatin.
Immunomodulatory activity of platinum compounds. The main recently reported aspects of the immunomodulatory activity of platinum compounds are shown.

because they are all capable of inducing IFN-β secretion. Indeed, induction of MHC I expression by a 24 h cisplatin exposure (6 µM followed by 72 h incubation in drug-free medium) occurs in parallel with IFN-β secretion by tumor cells [38].

Upregulation of MHC class I molecules has been described in human Non Small Cell Lung Cancer (NSCLC) cells exposed to concentrations of the combination cisplatin and vinorelbine with a growth inhibitory effect of around 50% or lower, but substantially ineffective on viability and therefore defined sub-lethal [39]. The levels of other cell surface molecules such as ICAM, Fas, MUC1 and carcinoembryonic antigen (CEA) were also enhanced by treatment in the studied tumor cell lines [39]. Tumor cell exposure to this combination made cells more sensitive to perforin/granzyme-mediated CTL killing, a phenomenon that is MHC-restricted because it was abolished by an HLA-A2 blocking antibody. Although the combination of cisplatin and vinorelbine was tested in this study without evaluation of the relative activity of each single agent per se, hence preventing the drawing of a conclusion on the specific effect of cisplatin; the available knowledge on this drug suggests that this Pt compound might display immunomodulating activities [39]. Of note, the concentration of cisplatin combined with 0.05 µM vinorelbine using a 6 h exposure was 1.6 µM and was selected based on the clinical data on unbound plasma peaks [40].

3.3. Additional immunostimulatory effects

Several lines of evidence support that low dose chemotherapy displays immunostimulatory effects. Such effects have been reported for conventional chemotherapeutic agents including cyclophosphamide, cisplatin and carboplatin [44, 45]. However, there is also evidence of immunostimulation when cisplatin is used at its maximum tolerated...
to the cytotoxic effect of CTLs specific to different antigens [46]. Under such conditions, there was no change in the expression of Fas or Fas ligand on tumor cells or splenocytes, whereas a marked increase in membrane cell permeability to granzyme B was observed; such an increase of granzyme B uptake was due to mannose-6-phosphate receptors, without requirement for perforin. Of note, such a mechanism allowed antigen-specific CTLs to kill both antigen-positive and antigen-negative tumor cells [51]. Besides, as mentioned above, exposure of NSCLC cells to the cisplatin-vinorelbine combination rendered tumor cells more sensitive to CTL-mediated killing [39]. Again, cisplatin is also capable to induce expression of Fas and ICAM-1 in human colon cancer cells in association with increased sensitivity to antigen-specific CTLs [52].

4. Concluding remarks

There has been a large gain in knowledge from the original reports on ICD regarding the immunological effects of chemotherapy, including Pt-based drug treatment. Many molecular details of ICD have been clarified including the mechanisms that are crucial for ICD induction. Thus, it is clear that cisplatin generally fail to induce ICD by itself, but a propensity towards ICD induction can be restored for this cytotoxic drug using ER targeting compounds or approaches [18, 22, 25, 30]. Besides, in spite of a non-immunogenic behavior, cisplatin appears to be endowed with an array of immunomodulatory activities that are already being explored and explored in the clinical setting, as shown by the use of immune checkpoint blockers (e.g., pembrolizumab) [53]. Early and recent evidence supports that chemotherapy, including Pt-based treatment can enhance the efficacy of immunotherapy [53]. For instance, cisplatin, carboplatin and oxaliplatin, by relieving immunosuppressive networks (i.e., producing evasion of antitumor immunity by PDL-1 and PDL-2) [54], can somehow increase the direct antitumor effects elicited by the inhibition of DNA functions [37].

In spite of the exciting results obtained in preclinical models, it remains to be defined if the drug concentrations used in that context can be achieved in vivo, particularly in the clinical setting. In fact, some of the effects reported for cisplatin on human monocyte-derived dendritic cells were obtained with cell exposure to micromolar concentrations (i.e., 25 μM) with long-term treatment (6 days) [55]. This leads to increase in the immunostimulatory ability of monocytes via IFN-β production as cisplatin-treated monocytes enhanced T cell proliferation, but it seems unlikely that these concentrations are achieved in vivo [55].

A novel promising aspect of the pharmacology of platinum compounds concerns the drug development side, particularly the emerging interest towards novel Pt pro-drugs with immune-modulating effects [56]. Although encouraging results regarding the immunostimulatory properties of Pt drugs are shown in clinical studies [53, 57], focused experimental efforts will be necessary to determine the specific contribution of each drug in terms of immune-stimulatory ability when combinations of chemotherapeutic agents are used. The molecular characterization of clinical tumors is already useful to optimize treatment and further improvement may be achieved. Indeed, tumors characterized by DNA MMR defects and MSI (including cisplatin-resistant tumors) might be more immunogenic than others and thereby more responsive to immune checkpoint inhibitors [31, 57]. A better definition of the molecular mechanisms underlying the effects of Pt drugs on the immune system may also be helpful in understanding the side effects of these cytotoxic com-
pounds because a patho-physiological role for specific populations of immune cells has been described [58].

**Authors’ contribution**

CC and PP wrote the manuscript. All authors read and approved the final manuscript.

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**Conflict of Interest**

Authors declare no conflict of interest.

**References**


