Cisplatin Toxicity Is Mediated by Direct Binding to Toll-Like Receptor 4 through a Mechanism That Is Distinct from Metal Allergens

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ABSTRACT
Cisplatin is an effective chemotherapeutic agent, yet its use is limited by several adverse drug reactions, known as cisplatin-induced toxicities (CITs). We recently demonstrated that cisplatin could elicit proinflammatory responses associated with CITs through Toll-like receptor 4 (TLR4). TLR4 is best recognized for binding bacterial lipopolysaccharide (LPS) via its coreceptor, MD-2. TLR4 is also proposed to directly bind transition metals, such as nickel. Little is known about the nature of the cisplatin-TLR4 interaction. Here, we show that soluble TLR4 was capable of blocking cisplatin-induced, but not LPS-induced, TLR4 activation. Cisplatin and nickel, but not LPS, were able to directly bind soluble TLR4 in a microscale thermophoresis binding assay. Interestingly, TLR4 histidine variants that abolish nickel binding reduced, but did not eliminate, cisplatin-induced TLR4 activation. This was corroborated by binding data that showed cisplatin, but not nickel, could directly bind mouse TLR4 that lacks these histidine residues. Altogether, our findings suggest that TLR4 can directly bind cisplatin in a manner that is enhanced by, but not dependent on, histidine residues that facilitate binding to transition metals.

SIGNIFICANCE STATEMENT
This work describes how the xenobiotic cisplatin interacts with Toll-like receptor 4 (TLR4) to initiate proinflammatory signaling that underlies cisplatin toxicities, which are severe adverse outcomes in cisplatin treatment. Here, this study provides a mechanistic bridge between cisplatin extracellular interactions with TLR4 and previous observations that genetic and chemical inhibition of TLR4 mitigates cisplatin-induced toxicity.

Introduction
Cisplatin is the oldest and most potent of the platinum-based chemotherapeutics available (Rosenberg et al., 1965; Rosenberg and VanCamp, 1970). It is used to treat a variety of solid-state cancers—ranging from head-and-neck to ovarian and testicular (Einhorn, 2001; Low et al., 2012; Bookman, 2016; de Vries et al., 2020; Muzaffar et al., 2021; Nagasawa et al., 2021). Consisting of two amine ligands and two chloride ions bound to a platinum core, cisplatin mediates its antitumor effects by intercalating into the DNA of replicating cells. Cisplatin binding to DNA inhibits strand separation and DNA damage repair, leading to the buildup of DNA damage that eventually leads to cell death (Dasari and Tchouwou, 2014). Treatment regimens that include cisplatin can have 5-year overall survival rates of up to 90%, making it integral to cancer therapy.

Despite its effectiveness, cisplatin use has been limited by the discovery of cisplatin-induced toxicities (CITs) (Tsang et al., 2009; El-Awady et al., 2011; Shahid et al., 2018). The development of CITs such as nephrotoxicity, peripheral neurotoxicity, and ototoxicity appears to be dependent on both the dosages administered and the age of patients. Children appear to be a highly susceptible population, particularly to cisplatin-induced hearing loss (Kamalakar et al., 1977; Romano et al., 2020; Ruggiero et al., 2021). Newer platinum-based drugs were explicitly designed to reduce toxicities and overcome chemotherapy resistances (Kelland, 2007). Unfortunately, most modifications made to the underlying structure of cisplatin come at the cost of its therapeutic efficacy—leaving cisplatin the primary choice for treatment and underscoring the need to develop mitigations for its toxicities. Studies of the underlying mechanisms of CITs have revealed both unique features, such as anatomic location, and shared features among CITs. Inflammation has proven to be a critical aspect of CITs (Domingo et al., 2022). The inhibition of direct and indirect mediators of inflammation has proven to be effective in mitigating CITs in vitro and in vivo preclinical studies. (Rybak et al., 1999, 2009; So et al., 2007; Kaur et al., 2011; Kim et al., 2011; Babolmorad et al., 2021; Domingo et al., 2022).

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ABBREVIATIONS: CIT, cisplatin-induced toxicity; DAMP, damage-associated molecular pattern; HEK, human embryonic kidney; hTLR4, human Toll-like receptor 4; IL, interleukin; Kd, estimated (apparent) binding affinity; LPS, lipopolysaccharide; MD-2, myeloid differentiation factor 2; MST, microscale thermophoresis; mTLR4, mouse Toll-like receptor 4; TLR4, Toll-like receptor 4.
Models of CIT where proinflammatory signaling systems, such as the Toll-like receptor 4 (TLR4) pathway, have been removed or otherwise disabled have also exhibited resistance to cisplatin toxicity (Ramesh and Reeves, 2003; Tsuruya et al., 2008; Zhang et al., 2008; Park et al., 2014; Zhang et al., 2014; Woller et al., 2015; Zhou et al., 2018, 2020; Li et al., 2019; Gao et al., 2020; Babolmorad et al., 2021; Zhang et al., 2022).

TLR4 is a membrane-bound pattern-recognition receptor typically responsible for recognizing and mounting proinflammatory innate immune responses to both pathogen-associated molecular patterns and damage-associated molecular patterns, DAMPs (Kawai and Akira, 2006). TLR4 exists in complexes with coreceptors that can help dictate its specificity. The best characterized agonist of TLR4 is bacterial lipopolysaccharide (LPS), which is bound to TLR4 in conjunction with the coreceptor myeloid differentiation factor 2 (MD-2). The current understanding of TLR4 activation suggests that TLR4 itself contains features that allow for the binding of various potential agonists—with coreceptors providing support to complete the TLR4 dimerization necessary for downstream signaling (Kawasaki and Kawai, 2014). Agonists interact with TLR4 through its extracellular/ectodomain. The ectodomain region contains unique amino acid residue combinations that, in human TLR4, confer a greater affinity for hexa-acetylated LPS versus penta-acetylated LPS compared with that of murine TLR4 (Hajjar et al., 2002). Certain single nucleotide polymorphisms can also confer LPS-specific TLR4 hypoactivation (Richard et al., 2021). Similarly, histidines 456 and 458 in the TLR4 ectodomain were found to mediate the interaction of TLR4 with group 9 and group 10 transition metals, leading to contact allergen hypersensitivities (Schmidt et al., 2010; Raghavan et al., 2021). Metal agonist treatment concentrations were also specifically chosen to ensure that they retained solubility in media.

TLR4 Histidine-456 and Histidine-458 Mutisite-Directed Mutagenesis. TLR4 histidine residues 456 and 458 were replaced with alanine and leucine, respectively, by site-directed mutagenesis according to the manufacturer protocols (Agilent, cat. #200514/200515). The mutagenic primers were 5′-TACCTTGACATTTCGTACCTCTCAGGAGTTGCTTTCAATGC-3′ and 5′-GCCATGTGAAGCAACTCTCGTGAGAAGTACGAAATGGC-3′. TLR4 mutations were confirmed by Sanger sequencing using primers 5′-TTGGGACACAGGCTTAAAC-3′ and 5′-GAGGAGTCCAGGAACTGCAAA-3′.

Immunoblotting. Transfected cells were collected and lysed using 400 μL of Pierce RIPA Lysis and Extraction Buffer (Thermo Scientific, cat. #89800) containing Pierce protease inhibitors (Thermo Scientific, cat. #A32953). To lyse cells, cells were kept on ice and scraped after 15 minutes of exposure to lysis buffer. Samples were heated at 80°C for 10 minutes, and protein was separated by SDS-PAGE prior to transfer to nitrocellulose membrane. Membranes were probed overnight with mouse anti-hemagglutinin (1:2500) (Santa Cruz, 12CA5 sc-57592) or mouse anti-glyceroldehyde-3-phosphate dehydrogenase (L5000) (Invitrogen, MA5-15738) and then probed for 1 hour with goat anti-mouse secondary antibody (1:5000) (LiCor, IRDye 800CW). Probed membranes were imaged on a LiCor Odyssey, and the immunoblotting procedure was performed according to their recommendations.

Cell Transfections. HEK293-Null2 cells were transfected with either an empty vector control, human TLR4 expression clone (kindly provided by Dr. A. Hajjar, Cleveland Clinic), or TLR4 variant expression clone. To assess the impact of histidine variants on TLR4-mediated immune responses to LPS, HEK293-Null2 cells were also cotransfected with a human MD-2 expression clone (OriGene, RC204686). JetPRIME (Polyplus, C89129-924) reagent was used for all transfections in accordance with manufacturer specifications.

ELISA and Cell Viability Assays. IL-8 secretion was used as a measure of TLR4 activation in HEK-null2 and HEK-hTLR4 cells and was quantitated through ELISAs (88-8088, Invitrogen) as recommended by the provider (Invitrogen). Secreted cytokines were collected 48 hours post-agonist treatment and quantified by ELISA following the manufacturer protocols. Protein secretion was normalized to cell viability to account for the differing toxicities of agonists.

Briefly, 96-well plates were coated with anti–interleukin (IL)-8 antibodies and used to capture IL-8 present in sample supernatants. A secondary anti-IL-8 antibody conjugated with biotin was used to immobilize streptavidin–horseradish peroxidase enzymes that oxidized 3,3′,5,5′-tetramethylbenzidine substrate to produce a colorimetric signal. The signal was quantitated using a SpectraMax i3x plate reader (Molecular Devices), and IL-8 concentrations were derived from manufacturer-provided standard curves.

Cell viability was measured using MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (ACROS, 158990010) for the purpose of normalizing ELISA data. MTT was added to cells posttreatment at 1 mg/mL and incubated for 4 hours. The absorbance of solubilized formazan was measured at 590 nm on a SpectraMax i3x plate reader (Molecular Devices).

Microscale Thermophoresis. The NanoTemper Microscale Thermophoresis Monolith system was used to measure normalized fluorescence changes associated with protein–ligand binding. Soluble recombinant TLR4 (human and mouse) and TLR4 agonists were prepared, separately, in PBS containing 2% dimethylsulfoxide and 0.5% Tween-20, except for microscale thermophoresis (MST) experiments involving nickel.
Nickel MST experiments were performed with reagents resuspended in 2% dimethylsulfoxide and 0.1% Pluronic F-127 PBS buffer. Serial dilutions of agonists were mixed 1:1 with solutions of hTLR4 (100 µg/mL) or mTLR4 (100 µg/mL). Binding assays were performed using NanoTemper NT.LabelFree instrument (NanoTemper Technologies). All experiments were carried out at room temperature in hydrophobic capillaries with 20% light-emitting diode power (fluorescence lamp intensity) and 40% microscale thermophoresis power (infrared laser intensity). Microscale thermophoresis data were analyzed by Monolith Affinity Analysis v.2.2.6 software. Binding affinities and associated statistics reported are estimates from GraphPad Prism.

**Results**

**Soluble Recombinant TLR4 Can Reduce Cisplatin-Induced TLR4 Activation in Vitro.** Platinum (II) ions, platinum (IV) ions, and cisplatin have all been shown to elicit TLR4-dependent proinflammatory cytokine secretion (Babolmorad et al., 2021). Although these interactions appeared to be independent of MD-2, their molecular nature remains to be elucidated. Metal allergens, such as nickel, can elicit similar inflammatory signaling via direct activation of TLR4 (Schmidt et al., 2010; Raghavan et al., 2012; Rachmawati et al., 2013; Oblak et al., 2015). Notably, this can be blocked by soluble TLR4. Accordingly, we sought to determine whether soluble forms of TLR4 could block cisplatin activation of TLR4.

HEK293 cells stably expressing hTLR4, MD-2, and cluster of differentiation 14 (CD14) (HEK293-hTLR4) were pretreated for 1 hour with 0.1 nM soluble hTLR4 or mTLR4 and then treated with either no agonist, 1 ng/mL LPS, 200 µM nickel, or 25 µM cisplatin for 48 hours prior to measuring cell viability and proinflammatory cytokine (IL-8) secretion as described in Methods. Residual TLR4 activity was calculated as the percentage of normalized IL-8 secretion obtained in the absence of soluble TLR4 treatment. Differences in residual TLR4 activity were assessed by two-way ANOVA with Bonferroni multiple-testing correction.

LPS activation of TLR4 could not be blocked by either soluble hTLR4 (Fig. 1A) or mTLR4 (Fig. 1B). This was expected since LPS binding to TLR4 requires MD-2. By contrast, soluble hTLR4, but not soluble mTLR4, reduced proinflammatory response to nickel by 25% (Fig. 1, A and B). This was expected because mTLR4 lacks the critical histidine residues found in hTLR4 necessary for binding group 10 metal allergens (Schmidt et al., 2010). Notably, both soluble mTLR4 and hTLR4 could inhibit TLR4 activation by cisplatin in this assay by approximately 50%. These data support a model where cisplatin binding to TLR4 does not require coreceptors.

**Metal Allergens and Cisplatin Can Directly Bind hTLR4 without Additional Cellular Components.** Our experiments with soluble recombinant TLR4 indicate that TLR4 coreceptors are not required to block nickel- and cisplatin-induced TLR4 signaling, but they do not confirm a direct interaction between these ligands and TLR4. To rule out the possibility that soluble TLR4 was binding (and blocking) endogenous TLR4 or DAMPs released in response to cellular toxicity, we attempted to detect direct binding events using recombinant protein via MST.

To validate this system, soluble recombinant forms of hTLR4 were exposed to increasing concentrations of LPS without MD-2. The range of LPS concentrations chosen (0.009–2.5 µM) was based on estimated binding affinities associated with TLR4:MD-2 complexes (Viriyakosol et al., 2001). No detectable binding was observed between hTLR4 and LPS in this assay (Fig. 2A), as evidenced by the lack of curve fit to a three-parameter agonist binding model. By contrast, nickel has been demonstrated to be a TLR4-dependent metal contact allergen, so we reasoned it could be a positive control for our MST studies, though we are not aware of any methods that have shown direct binding of nickel to TLR4. In line with previous in vitro reports, our MST data suggested that hTLR4 bound nickel within the tested range of 0.49–500 µM. The curve fit to a three-parameter agonist binding model was R2 = 0.695, suggesting that the Kd of 2.72 µM (95% confidence interval, 0.736–7.71 µM) supports a direct binding model of nickel to human TLR4.

To examine the specificity of our MST assay for detecting soluble TLR4 binding events, we tested calcium chloride as a negative control for metal binding to TLR4. Calcium is a group 2 metal, and we are not aware of any reported calcium interactions with TLR4, so we did not expect to observe binding interactions between TLR4 and calcium. Unlike nickel, hTLR4 displayed little to no capacity to bind calcium within the concentration range of 0.49–500 mM in our assay (Fig. 2C), with a curve fit R2 = 0.3 to the agonist binding model. This contrasts with previous reports showing that calcium binding to calmodulin is robustly detected by MST (Wienken et al., 2010; Seeger et al., 2017).

We next tested cisplatin in the MST assay within the concentration range of 0.73–1665 µM and detected clear binding of cisplatin to hTLR4, with a strong curve fit of R2 = 0.928 to the agonist binding model (Fig. 2D). The apparent Kd of this interaction was 50.57 µM (95% confidence interval of 38.43–66.76 µM). Pt (II) and Pt (IV) solubility were incompatible with the MST buffer system, and this precluded our ability to test their binding to hTLR4.

**TLR4 Activation by Platinum and Cisplatin Is Enhanced by Known Metal-Binding Residues.** To further understand the interaction between hTLR4 and cisplatin, we used site-directed mutagenesis to replace histidines 456 and 458 (located within the extracellular region of hTLR4 that is part of soluble hTLR4) with alanine and leucine, respectively. These histidines have been reported to be critical mediators of nickel-TLR4 binding (Schmidt et al., 2010; Raghavan et al., 2012). Hemagglutinin epitope-tagged hTLR4 variant constructs were transiently expressed in HEK293-Null2 cells (which do
not express endogenous hTLR4, MD-2, or CD14 prior to treatment with LPS, nickel, platinum (II), platinum (IV), or cisplatin. Transiently expressed hTLR4 levels were assessed by immunoblotting, which revealed that hTLR4 wild type and hTLR4 H456A-H458L levels were comparable, indicating that these variants do not induce gross destabilization of hTLR4 (Fig. 3A). Further supportive of this conclusion, no difference in IL-8 secretion was observed between wild-type and variant TLR4 when stimulated with 1 ng/mL LPS in the presence of MD-2 (Fig. 3B). As expected, the hTLR4 H456A-H458L variant was impaired in

![Fig. 1. Soluble recombinant TLR4, TLR4(s), can inhibit TLR4-activated proinflammatory IL-8 secretion to distinct agonists in HEK293 hTLR4 cells.](image)

(A) IL-8 secretion of HEK293-hTLR4 cells pretreated with 0.1 nM soluble recombinant human TLR4 and subsequently treated with either 1 ng/mL LPS, 200 μM Ni²⁺, or 25 μM cisplatin. Data are shown as the percentage of TLR4 activity in the absence of soluble hTLR4. Nil representative of cells treated without agonists (n = 4 independent biologic replicates for all conditions). (B) IL-8 secretion of HEK293-hTLR4 cells pretreated with 0.1 nM soluble recombinant mouse TLR4 and subsequently treated with either 1 ng/mL LPS, 200 μM Ni²⁺, or 25 μM cisplatin. Data are shown as the percentage of TLR4 activity in the absence of soluble mTLR4 (n = 4 for all conditions). Nil representative of cells treated without agonists. For all panels, all individual data points (technical replicates) from each experiment are plotted with a central bar for the mean and whiskers denoting S.D. Statistical analyses were performed through two-way ANOVA and Bonferroni multiple-testing correction. ****P < 0.0001. ns, not significant.

![Fig. 2. Human TLR4 can directly bind nickel and cisplatin. Microscale thermophoresis analysis showing normalized fluorescence of hTLR4 plotted against the indicated concentrations of](image)

(A) LPS (n = 5 independent replicates), (B) Ni²⁺ (n = 3 independent replicates), (C) Ca²⁺ (n = 6 independent replicates), or (D) cisplatin (n = 3–6 independent replicates), respectively. For all panels, data fitted to nonlinear best-fit curves following three parameters with a standardized slope. (A) Apparent Kₐ unavailable/undetectable. (B) Apparent Kₐ = 2.72 μM, R² = 0.689. (C) Apparent Kₐ unavailable/undetectable. (D) Apparent Kₐ = 50.57 μM, R² = 0.927.
IL-8 secretion in response to 200 μM nickel compared with wild-type hTLR4 (Fig. 3C). These data indicate that although not impacting global TLR4 structure, the histidine variants do impair metal activation of hTLR4. Interestingly, the histidine variant TLR4 had differential effects on responses to platinum (II), platinum (IV), and cisplatin. Similar to the nickel phenotype, the replacement of His456/458 completely abrogated IL-8 secretion in response to 100 μM platinum (II) (Fig. 3D). By contrast, His456/458 replacement only partially reduced IL-8 secretion induced by 100 μM platinum (IV) (Fig. 3E) and 25 μM cisplatin (Fig. 3F). Here, variant TLR4 activation was reduced fivefold in response to platinum (IV) and 2.5-fold in response to cisplatin while retaining significant IL-8 secretion compared with the negative control (empty vector). Taken together, the data suggest that the direct interactions between cisplatin and hTLR4 occur, in part, due to the metal-binding properties intrinsic to hTLR4.
Cisplatin and Nickel Use Distinct Mechanisms to Bind TLR4. Previous investigations have identified critical differences in how mTLR4 and hTLR4 interact with potential agonists. For example, mTLR4 does not contain histidines at conserved positions 456 and H458. Our blocking experiments with soluble mouse TLR4 and the differential impact of His456 and His458 mutation on TLR4 activation by nickel and cisplatin hinted at differences in the ways these agonists interact with TLR4. To further explore this concept, we investigated nickel and cisplatin binding to soluble mouse TLR4 in the MST assay. Unlike our experiments with human TLR4, we were unable to detect binding of nickel to mouse TLR4 within the same range of concentrations (0.49–500 μM) by MST as no curve could be fit to the agonist binding model (Fig. 4A).

By contrast, mouse TLR4 was able to bind cisplatin within a similar range of tested concentrations (0.29–1665 μM) as indicated by a curve fit of $R^2 = 0.721$. The interaction of cisplatin and mouse TLR4 in the MST assay occurred with a lower apparent affinity $K_d = 104.9 \mu M$ (95% confidence interval of 52.24–205.5 μM) compared with hTLR4.

To determine if these affinities were appreciably different, we

Fig. 4. Mouse TLR4 directly binds cisplatin but not nickel. Microscale thermophoresis analysis showing normalized fluorescence of mTLR4 plotted against the indicated concentrations of (A) Ni$^{2+}$ ($n = 3$ independent replicates) or (B) cisplatin ($n = 3$ to 4 independent replicates). (A) Apparent $K_d$ unavailable/undetectable. (B) Apparent $K_d = 104.9 \mu M$; $R^2 = 0.721$.

Fig. 5. TLR4 is activated by LPS, nickel, and cisplatin by distinct mechanisms. (Left panel) LPS requires MD-2 for TLR4 activation and cannot directly bind soluble TLR4 species. (Middle panel) Nickel can directly bind human TLR4, and this requires His456/His458. Soluble hTLR4 can block nickel activation of TLR4 (denoted by black line). Nickel cannot bind to mouse TLR4, which lacks these histidine residues. (Right panel) Cisplatin can directly bind both human and mouse TLR4, and this is not strictly dependent on His456/His458. Cisplatin has higher affinity for soluble hTLR4, which is more effective at blocking cisplatin activation of TLR4 compared with soluble mTLR4 (denoted by solid black versus gray dashed lines). The additional TLR4 residues that contribute to cisplatin binding remain to be elucidated (denoted by “?”). Figure was created with BioRender.com.
normalized the MST data to “fraction bound” and compared the curve-fit parameters (Supplemental Fig. 1). These analyses showed that affinity of cisplatin significantly increased by ~2.5-fold for hTLR4 compared with mTLR4 \( (P < .0002) \).

Discussion

The connection between TLR4 and the development and severity of CITs has been a key area of investigation. TLR4 has been linked molecularly to CITs; TLR4 activation can induce the production of reactive oxygen and nitrogen species, apoptosis, and proinflammatory signaling pathways, e.g., NF-κB. Yet, the molecular details of this linkage were unclear.

Initial models suggested that DNA damage generated by cisplatin could elicit the release of DAMPs that could then be subsequently detected through pattern-recognition receptors such as TLRs (Miller et al., 2010; Manohar and Leung, 2018). The relevance of TLR4-specific DAMPs to CIT development remains inconclusive, however. Cisplatin has minimal effects on TLR4-specific DAMP expression (Zhang et al., 2008), and in our previous work, we established that DAMP signaling can be differentiated from cisplatin-induced TLR4 activation since TLR4 activation in response to HMGB1 depended on the MD-2 coreceptor, in contrast to cisplatin (Babolmorad et al., 2021). Alternatively, others have posited that cisplatin-induced activity was dependent on synergies with other TLR4 ligands, such as LPS (Oh et al., 2011).

This work uncovers new molecular insights on the interaction between cisplatin and TLR4, supporting a role for direct activation of TLR4 by cisplatin. Our use of microscale thermophoresis with recombinant soluble TLR4 protein removes confounding factors normally associated with working with cell-based assays. Using MST, we have shown that TLR4 can bind nickel, as implied in the literature, and also cisplatin, without the need for any additional cellular factors, e.g., MD-2. Nevertheless, although these findings indicate that cisplatin is sufficient to bind and activate TLR4, it does not rule out the involvement of other TLR4 agonists, e.g., LPS or DAMPs, that may contribute to TLR4 activation during CITs in vivo.

Our studies also identified intriguing distinctions in how mTLR4 and hTLR4 bind cisplatin and other metal agonists that requires further characterization. Our work confirmed that histidines 456 and 458 of human TLR4 are critical for activation by nickel but their contribution to activation by cisplatin was less definitive, since replacement of these residues retained considerable activity. It should be noted that although we interpreted the activation of hTLR4 H456A-H458L by LPS as an indication that these variants did not create global perturbations in TLR4 structure, we cannot rule out the fact that MD-2 may have stabilized general perturbations induced by histidine replacement. Nevertheless, one explanation for the residual activation of His variant TLR4 by cisplatin, as well as the direct binding of cisplatin to mTLR4, is that additional residues that are conserved in human and mouse TLR4 contribute to cisplatin binding. One candidate is His431, a residue known to contribute to metal-induced hypersensitivities (Schmidt et al., 2010).

Our evidence of direct mTLR4-cisplatin binding justifies follow-up studies to evaluate the role of H431 in cisplatin binding to mTLR4 and hTLR4. H431 (but not H456/458) also exists on the zebrafish tlr4bb gene. We previously showed that zebrafish neuromasts, structurally analogous to human ear hair cells, are susceptible to cisplatin-induced toxicity in a tlr4-dependent manner (Babolmorad et al., 2021).

Future work should seek to better characterize the chain of events that follow from direct TLR4 binding. Metal agonists are known to induce TLR4 homodimerization and elicit TLR4 downstream signaling and activation differently, with some only capable of facilitating homodimerization without activation compared with others (Raghavan et al., 2012). Cisplatin, platinum ions, and perhaps other platinum-based agonists may thus interface with TLR4 to induce homodimerization and activation to different degrees.

In summary, our data provides evidence for direct TLR4-cisplatin binding interactions that are enhanced by, but are not dependent on, TLR4 metal-binding interactions. This is reinforced by our prior work wherein we showed that both mTLR4 and hTLR4 were mediators of cisplatin-induced ototoxicity that can be chemically targeted to mitigate cisplatin toxicity. Moreover, our findings argue for distinct mechanisms of TLR4 activation by cisplatin, nickel, and LPS (Fig. 5). This raises the attractive opportunity of selectively inhibiting TLR4 activation rather than ablating its function entirely. A precision treatment to reduce cisplatin-induced toxicities while preserving TLR4 bacterial detection would be an ideal otoprotectant to optimize the safety of cisplatin treatment.

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Authorship Contributions

Participated in research design: Domingo, Groenendyk, Michalak, Bhavsar.

Conducted experiments: Domingo.

Contributed new reagents or analytic tools: Michalak, Bhavsar.

Data analysis: Domingo, Groenendyk, Michalak, Bhavsar.

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