Improving chemotherapeutic regimens by mitigating hearing loss as a limiting side-effect and examining transition metal ions as a ligand for zebrafish Tlr4

by

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ABSTRACT

Cancer is often a debilitating disease associated with the uncontrolled growth of cells within an organism. We have made significant strides over the past few decades in treating many cancers, unfortunately, many of these treatments have unfavourable side effects. Cisplatin is a revolutionary drug in cancer treatment that contributes to an approximately 80% five-year survival rate in pediatric patients, but its use is limited by several adverse side effects, particularly ototoxicity. Cisplatin-induced ototoxicity (CIO) is permanent, bilateral, sensorineural hearing loss found in up to 90% of patients treated with cisplatin. There are currently very few treatments to mitigate CIO, and only one is approved by the FDA. Our collaborators in the Bhavsar Lab have recently determined a novel pathway through which cisplatin contributes to the generation of CIO and have demonstrated its potential as a therapeutic target.

Toll-like receptor 4 is the first discovered innate immune pattern recognition receptor. It is crucial in signalling to the body when to initiate an inflammatory response following a gram-negative bacterial infection. The function of TLR4 in mammals is well characterized, but its function in other species, particularly fish, is still under debate. A major problem with understanding its function arises in the confusion behind its origins. Not all living things bind to the same TLR4 ligand, and in many cases, we are still unsure of what it binds to entirely. TLR4 was recently determined to bind to nickel and other heavy metals to induce a proinflammatory response and mediate an allergic response. Furthermore, it has recently been shown to bind to the platinum-containing drug cisplatin directly, mediating the generation of CIO. Due to the unique binding mechanism of these metals to TLR4, we believe it would be possible to selectively inhibit cisplatin binding while maintaining the regular immune function of TLR4. This novel

mechanism would distinguish it from several CIO therapies, demonstrating its promise for treating CIO.

Zebrafish make a great animal model for disease and share greater than 80% of disease-related genes with humans. Their small size, optical transparency, and high throughput nature also provide an advantage over other model systems. Zebrafish are known to possess three TLR4 paralogs, Tlr4ba, Tlr4al, and Tlr4bb, but like many fish, their function and origins remain controversial. However, their utility in research related to CIO is still particularly valuable due to the presence of hair cell clusters on the exterior of their bodies called neuromasts. These hair cells are homologous to the hair cells within the inner ear of mammals. Their position and similarity make them an excellent model for CIO and particularly suited for novel therapeutic drug discovery.

This thesis aims to tease apart some of the uncertainties in the evolutionary history of TLR4 while advancing our understanding of its activities in zebrafish. I also aim to utilize zebrafish as an effective model of CIO to screen novel selective therapeutics. To achieve these objectives, we created larval genetic mutants of Tlr4, allowing us to test its interaction with specific ligands in vivo. By creating a heterologous cell system using zebrafish Tlr4, we extended these findings in vitro. Finally, by creating a novel vibration-based behavioural assay, we demonstrated the end-point effects of a novel therapeutic capable of selectively inhibiting Tlr4 to mitigate CIO. The research presented in this thesis establishes the foundation for future investigations that aim to selectively inhibit TLR4 in order to reduce CIO while identifying potential ligands for a presently orphaned receptor.

PREFACE

This thesis is an original work by Aaron P. D. Fox. Approval for this work was granted by the University of Alberta Animal Care and Use Committee and follows the Canadian Council on Animal Care (CCAC). The author has completed mandatory training for animal users offered by the University of Alberta and as directed by the CCAC.

Chapter 2 is being prepared as a manuscript. At the time of writing, this manuscript is still under revision, and was written by APDF, with editing contributions from WTA. Tracy Lee from the Bhavsar lab was responsible for the in vitro cell culture work. Christie Li generated gRNA sequences as well as PCR primers for crispant validation.

Some of the research conducted in Chapter 3 is being prepared as a collaborative manuscript between the Bhavsar lab led by Dr. Amit P. Bhavsar at the University of Alberta, the Berman lab led by Dr. Jason Berman at the University of Ottawa, and the West lab led by Dr. Frederick G. West at the University of Alberta. At the time of writing, this manuscript in still being compiled, but this chapter was written by APDF with editing contributions from WTA. All in vivo work presented in this thesis was generated by APDF. Novel derivatives were generated by the West lab. The heterologous cell culture work was generated by Tracy Lee. Sakina Mithaiwala was instrumental in the generation of the YO-PRO1 assay and in helping establishing creation of the crispant mutant larva. Niall Pollock was fundamental in conceptualization and early instruction of fluorescent staining assays. Summary Table 3.1 represents in vitro cell culture data collected by Asna Latif and Ghazal Babolmorad. Christie Li generated gRNA sequences as well as PCR primers for crispant validation.

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LIST OF ABBREVIATION

ACD	Allergic contact dermatitis
aLL	Anterior lateral line
ALR	Aim2-like receptors
AP-1	Activating protein-1
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein
BLB	Blood labrynth barrier
CARD	Caspase-recruitment domain
CD	Cluster differentiation
CIO	Cisplatin induced ototoxicity
CLR	C-type lectin receptor
CRISPR	Clustered regularly interspaced short palindromic repeats
CTR	Copper transport protein
DAMP	Damage associated molecular patterns
DASPEI	2-(4-(Dimethylamino)styryl)-N-ethylpyridinium iodide
DC	Dendritic cell
DD	Death domain
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPOAE	Distortion product otoacoustic emissions
DRG	Dorsal root ganglia
EHF	Extended high frequency
ERK	N-ethyl-N-nitrosourea
ETOH	Ethanol
EV	Empty vector
FDA	Food and drug administration
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
GSH	Glutathione
HCS	Highly conserved segment
HDMS	Hexamethyldisilazane
HEI-OC1	House ear institute-Organ of Corti 1
HEK	Human embryonic kidney
HMGB	High mobility group box 1
HR	Homologous recombination
HSP	Heat shock protein
ICL	Intercellular linker region
IFN	Interferon

IL	Interleukin
IRAK	Interleukine-1 receptor-associated kinase
IRF	Interferon regulatory factor
LBP	Lipid binding protein
LPS	Lipopolysaccharide
LRP	Lipoprotein receptor related protein
LRR	Leucine-rich repeat
LRRCT	LRR C-termini
LRRNT	LRR N-termini
MAMP	Microbial associated molecular pattern
MD	Myeloid differentiation
MMR	Mismatch repair
MRP	Multidrug resistance protein
MYA	Million years ago
NAC	N-acetylcysteine
NER	Nucleotide excision repair
NET	Neutrophil extracellular traps
NF	Nuclear factor
NK	Natural Killer
NLR	Nucleotide oligomerization domain-like receptor
NO	Nitric oxide
NOX3	NADH oxidase 3
OAT	Organic anion transporter
OCT	Organic cation transporter
OHC	Outer hair cell
PAMP	Pathogen associated molecular pattern
pLL	Posterior lateral line
PRR	Pattern recognition receptor
PYD	Pyrin domain
RLR	Retinoic acid-inducible gene-1-like receptors
RNP	Ribonuclease complex
ROS	Reactive oxygen species
SEM	Scanning electron microscope
SMOC	Supramolecular organizing centre
STS	Sodium thiosulfate
SV	Stria vascularis
TALEN	transcription activator-like effector nuclease
TILLING	Targeting induced local lesions in genome
TIR	Toll/IL-1 receptor
TIRAP	Toll/interleukin-1 domain-containing adaptor protein
TRIF	TIR domain-containing adaptor protein inducing IFNβ
TLR	Toll-like receptor

TMD	Trans-membrane domain
TNF	Tumour necrosis factor
TRAF	Tumour necrosis factor receptor associated factor 6
TRAM	TRIF-related adaptor molecule
TRP	Transient receptor potential
VS	Variable segment
WT	Wildtype

Chapter 1. INTRODUCTION

Chemotherapeutics are a mainstay therapy in cancer treatment; however, due to their toxicity, treatment often leads to unwanted adverse side effects. This chapter will begin by briefly describing some of the most common chemotherapeutics used in cancer treatment, followed by the challenges associated with using one of the most common anticancer drugs, cisplatin. I will then describe how zebrafish can be utilized as an excellent model in cancer therapy and their role in drug discovery. Following the acute observation that toll-like receptor 4 can be utilized as a therapeutic target for cisplatin-induced ototoxicity, I will describe why it is a plausible target in zebrafish for testing novel drugs to reduce this undesirable side effect.

1.1 Chemotherapeutics in cancer treatment

Cancer is the second leading cause of death worldwide, with nearly 10 million deaths in 2020 (Behranvand et al., 2022; *World Health Organization: Cancer*, 2023). This disease is broadly defined by the uncontrolled growth of mutated cells within an organism with the possibility of spreading to other tissues. Cancer can arise from an interaction between four major factors: genetics, physical carcinogens (ex: UV light), chemical carcinogens (ex: alcohol), and biological carcinogens (ex: viruses). Cells are considered cancerous when mutations in their DNA lead to the inability to regulate their replication and division ((CCS), 2023). There are many different forms of cancer arising from a variety of different mutations, cell types, and environments. Unfortunately, this makes it nearly impossible to develop a universal treatment for all cancer types, however major strides have been made in the past few decades in treating this insidious disease.

Currently, there are nine major types of cancer treatment, including chemotherapy, hormone therapy, hyperthermia, immunotherapy, photodynamic therapy, radiation therapy, stem cell

transplants, surgery, and targeted therapy (*National Cancer Institute* 2023). Chemotherapy remains one of the primary cancer treatment methods due to its systemic application and effectiveness against metastasized cancers. Chemotherapy involves the use of drugs to stop or kill cancer cells. These drugs affect the rampant dividing nature of cancer cells by interacting with the DNA and can be further classified based on their specific mechanism of action (Bukowski, Kciuk, & Kontek, 2020). In the upcoming subsections, I will introduce one of the first and most used categories of chemotherapeutics, alkylating agents (N. C. I. (NIH), 2023). This type of chemotherapeutic will be reviewed to understand the mechanism behind these drugs, the underlying cause of their toxicity, and why cisplatin and other platinum-containing antineoplastics are unique in this category. Decades of research have attempted to improve upon cisplatin but have been somewhat unsuccessful in producing desired results. The history of alkylating agents will give context to the challenges behind drug discovery and the long and arduous process that has led to the current state of anticancer therapy. Furthermore, I will describe the evolution of platinum drugs and why cisplatin remains an effective choice in treating solid malignancies.

1.1.1 Alkylating agents

The first anti-tumour agent discovered was a DNA alkylating agent called sulphur mustard, which was discovered on accident by Dr. Cornelius Packard Rhoads after the Second World War (DeVita & Chu, 2008; Ralhan & Kaur, 2007). Alkylating agents have continued to evolve over the past seven decades, and today, they continue to be some of the most common and effective anticancer drugs. Traditionally, DNA alkylation is the attachment of an alkyl group to a DNA base, but recently, the term “alkylating agents” has been used to describe any compound that covalently attaches to the DNA (Bordin et al., 2013). For example, due to their DNA binding

capability, platinum-containing antineoplastics are often grouped in with other alkylating agents, although they do not alkylate DNA bases. This report will refer to alkylating agents in this manner, as many chemotherapeutics act on the DNA without the addition of alkyl groups. Alkylating agents are commonly used in combination with other chemotherapeutics and alone for treating hematological and solid malignancies (Lehmann & Wennerberg, 2021; Ralhan & Kaur, 2007). Alkylating agents exact their effects by attacking nucleophilic sites on DNA, covalently linking the drug to DNA, and preventing replication or transcription, causing mispairing and mutations, or fragmenting the DNA after DNA repair enzymes fail (Puyo, Montaudon, & Pourquier, 2014). Due to their electrophilic nature and mechanism of action, they can exact their effects at all phases in the cell cycle, but proliferating cells are more sensitive, particularly during DNA duplication. Since alkylating agents are highly effective, they also affect highly proliferative non-cancer cells. Therefore, searching for selective compounds capable of specifically targeting cancer cells would benefit therapeutic regimens.

Alkylating agents are either monofunctional or bifunctional, depending on their ability to react with one or two DNA strands (Ralhan & Kaur, 2007). Monofunctional alkylating agents prevent the action of essential processing enzymes from accessing the DNA, while bifunctional agents create either inter-strand (between two opposite strands) and/or intra-strand (within the same strand) cross-links. Alkylating agents will covalently bond to nucleophilic nitrogen, oxygen, and phosphate atoms within the DNA, particularly the N7-atom of guanine. Some other common sites for alkylation include the N1- and N3-atoms of adenine, the N3-atom of cytosine, and the O6-atom of guanine (Puyo, Montaudon, & Pourquier, 2014). Other less common targets include the phosphate backbone, some proteins and some other nitrogen and oxygen atoms within DNA bases. Classical alkylating agents primarily target the major groove of DNA, but

more recent derivatives were made to target the minor groove of DNA (Puyo, Montaudon, & Pourquier, 2014).

Classical alkylating agents can be further divided into five different categories based on their structure and mechanism action (Chiorcea-Paquim & Oliveira-Brett, 2023; Kashifa Fathima et al., 2022; Lehmann & Wennerberg, 2021; More et al., 2019). Nitrogen mustards, the original class of anticancer agents, are both monofunctional and bifunctional organic compounds marked by a nitrogen bound to two haloalkyl groups (Highley et al., 2022; Povirk & Shuker, 1994). Depending on the specific mustard, they form covalent bonds with either the DNA backbone or DNA bases. Another group of nitrogen-based alkylating agents, nitrosoureas, are unique in comparison to many alkylating agents due to their ability to cross the blood-brain barrier, making them valuable chemotherapeutics in treating certain brain tumours (Kaina & Christmann, 2019). They covalently bond to DNA by degrading into strong electrophiles under basic conditions. Alkyl alkane sulfonates are a smaller class of alkylating agents, with busulfan being the most representative drug in this category (Gate & Tew, 2011). Busulfan is a bifunctional, sulfonylated alkylating agent activated by the generation of carbonium ions capable of producing DNA cross-links as well as protein-DNA cross-links. Ethylenimines (aziridines) are characterized by aziridine cycles, a strained three-membered ring structure (Siddik, 2002). Their mechanism of action is the same as nitrogen mustards, except the aziridine cycles are not charged, making them less reactive compared to the haloalkyl groups. Lastly, triazenes are monofunctional alkylating agents with three adjacent nitrogen atoms called triazenyl groups (Marchesi et al., 2007). They mainly act on cells through the methylation of the O6-atom in guanine, with two alkylating agents, temozolomide and dacarbazine, currently used in a clinical setting.

1.1.2 Platinum (II) antineoplastics

Platinum-containing antineoplastics are a special class of drug often grouped in with alkylating agents, although they do not alkylate the DNA. They are considered alkylating agents because they act in a similar manner by binding and causing intra- and inter-strand DNA cross-links (Figure 1.1) (Parveen, 2022). The first platinum derivative used in cancer treatment was cisplatin (cis-diaminodichloroplatinum) discovered by Michele Pyrone in 1844. However, the initial discovery of this compound was not intended for cancer therapy. As for many great scientific discoveries, it was not until Barnett Rosenberg (1962) accidentally determined that platinum compounds produced by electrolysis inhibited bacterial cell division that the potential of metals as antineoplastic agents was considered (Rosenberg, Van Camp, & Krigas, 1965). It was later confirmed that pyrones chloride (now known as cisplatin) was the most potent agent, capable of recapitulating the finding of inhibiting bacterial cell division in its presence.

Following the approval of cisplatin for cancer treatment by the FDA in 1978, the search for other platinum complexes led to determining the structure-activity relationship required for platinum compounds to have antineoplastic activity. The structure-activity relationships resolved that the compounds should be neutral in charge, have a square-planar geometry, contain two cis amine ligands, and two cis anionic ligands (Cleare & Hoeschele, 1973). This initiated an extensive search for platinum compounds that fulfilled these rules and led to the discovery of carboplatin (cis-diammine(1,1-cyclobutanedicarboxylato)platinum) and oxaliplatin (R,R-cyclohexane-1,2-diamineoxalatoplatinum). The second generation of platinum chemotherapeutics, carboplatin, was approved for usage after testing by the National Cancer Institute by the FDA in 1989 and soon after, the third generation, oxaliplatin, was granted approval in 2002 (Ivanova, 2022). Fast forward to today, eight platinum-based anticancer agents

are approved for clinical use, with three approved for usage worldwide (Figure 1.2)(Alassadi, Pisani, & Wheate, 2022). Due to its ability to effectively enter cells, highly reactive nature, and efficacy in producing DNA damage, cisplatin remains the chemotherapeutic of choice in the treatment of germ cell tumours, bladder cancer, and head and neck cancers (Schoch et al., 2020; Szikriszt et al., 2020; Tsvetkova & Ivanova, 2022).

1.2 The chemotherapeutic Cisplatin

The discovery of cisplatin was a monumental innovation within cancer therapy and gained substantial recognition based on its ability to treat multiple solid malignancies, including testicular, ovarian, bladder, lung, cervical, head and neck cancer, etc. (Romani, 2022). The anticancer activity of cisplatin is primarily attributed to its ability to cross-link DNA, although other mechanisms such as oxidative stress, disruption of calcium homeostasis, and dysregulation of intracellular and extracellular proteins contribute to it's cytotoxicity (Dasari & Bernard Tchounwou, 2014; Makovec, 2019). Unfortunately, the usage of cisplatin is limited due to the development of resistance and toxic side effects. Resistance to cisplatin arises from a multitude of factors, including: reduced intracellular accumulation, inactivation by antioxidants, increased DNA repair, and alteration of proteins that contribute to inducing apoptosis (Siddik, 2003). Similarly, many factors contribute to the increased risk of cisplatin toxicity, which presents in many forms. These forms include, but are not limited to, nephrotoxicity, myelosuppression, neurotoxicity, gastrointestinal toxicity, and ototoxicity (Table 1.1)(Aldossary, 2019; Barabas et al., 2008; Qi et al., 2019). This section will briefly review cisplatin’s usage, resistance, toxicities, and current ototoxicity therapies with an overall emphasis on ototoxicity. This review will establish why we urgently need an effective therapy against cisplatin-induced ototoxicity and why current solutions fall short in this endeavour.

1.2.1 Usage of cisplatin and cellular mechanism of action

Understanding cisplatin's subsequent effects depends on comprehending how it is administered and develops into an active anticancer agent. Since the discovery of the first platinum anticancer agent, cisplatin, roughly half of all patients who receive chemotherapy are treated with a platinum drug (Armstrong-Gordon et al., 2018). Cisplatin is normally a yellow-orange crystalline powder reconstituted in sterile water and administered through slow intravenous infusion (Food and Drug Administration, 2019). Cisplatin dosage varies depending on the cancer type being treated, but in general, range from 20-100mg/m2 once a day, every 3-4 weeks (Ghosh, 2019). All platinum-based antineoplastics are considered prodrugs because they require the replacement of their chloride or carboxylate constituents with water to become active. This process is called aquation and is the rate-determining step in forming DNA adducts (Ahmad, 2017). Once cisplatin is in the bloodstream, the high concentration of chloride ions (~100mM) prevents cisplatin from immediately undergoing aquation (Qi et al., 2019). It is only once it enters the cells that the reduced concentration of chloride ions (~4-22mM) facilitates aquation. A large portion (>90%) of the cisplatin within the blood is also bound to plasma proteins resulting in its inactivation and excretion by the kidneys (Makovec, 2019; J. Wang et al., 2021). Only ~10% of cisplatin is free to be taken up into cells.

Cisplatin that enters cells is taken up through two main mechanisms: passive diffusion and active membrane transport. Passive diffusion is the primary mechanism of cisplatin uptake into cells and is facilitated by its neutral charge and small size, allowing for free passage through the nonpolar interior of the lipid bilayer (Eljack et al., 2014). Passive diffusion into cells is proportional to the extracellular concentration of cisplatin until ~3mM (Makovec, 2019). Active transporters such as copper transport protein 1 (CTR1) and organic cation transporter 1 and 2

(OCT1; OCT2) are reported to be involved in cisplatin uptake into the cell (Hu et al., 2017; McSweeney et al., 2021; Pabla et al., 2009). CTR1 is a ubiquitously expressed transport protein with higher expression within epithelial cells and connective tissue (Kuo et al., 2006). Organic cation and anion transporters are mainly expressed within the kidneys and/or the liver (Anzai, Kanai, & Endou, 2006; Cha et al., 2001; Hagenbuch & Meier, 2003; Wu et al., 1999). The exact mechanism behind cisplatin uptake by these transporters has yet to be demonstrated, and their involvement in its uptake is still poorly understood within the literature. Moreover, organic anion transporter 1 and 3 (OAT1; OAT3) have been implicated in cisplatin transport, toxicity, and resistance, but further study is required to elucidate their exact role (McSweeney et al., 2021; Nieskens et al., 2018; *World Health Organization: Cancer*, 2023).

Once within the cell, cisplatin forms two types of aquated derivatives: cis-[PtCl(NH3)2(OH2)]+ (monoaquated), and cis-[Pt(NH3)2(OH2)2]2+, which locks the majority of cisplatin within the cell due to their positive charge (Eljack et al., 2014). These derivatives are highly electrophilic and will readily react with sulfhydryl groups on proteins and nitrogen donor groups on nucleic acids. Only a small portion of the cisplatin (~1%) reaches the nuclear DNA, while the rest reacts with other biomolecules in the more immediate vicinity, such as cellular/membrane proteins and RNA (Yu, Megyesi, & Price, 2008). The binding of cisplatin to purine bases results in inter- and intra-DNA cross-links (adducts) (Figure 1.1). Cisplatin reacts most readily with the N7 position of guanine forming 1,2-intrastrand d(GpG) cross-links that make up to 90% of the adducts formed, while other bases are less preferable (Ghosh, 2019). These cross-links lead to the bending and unwinding of the DNA that can be recognized by high mobility group box 1 (HMGB1), forming a ternary complex. This complex prevents replication, leading to cell cycle arrest and apoptosis (Aldossary, 2019; Ghosh, 2019). DNA damage is also

recognized by other nuclear proteins, which leads to the activation of DNA repair machinery such as nucleotide excision repair (NER) and mismatch repair (MMR) that, if unsuccessful in repairing the DNA, leads to apoptosis (JOHNSTONE, PARK, & LIPPARD, 2014; Qi et al., 2019). Other signal transduction pathways are also activated in response to DNA damage, such as the p53 pathway, which also mediates cisplatin induced apoptosis (Brown, Kumar, & Tchounwou, 2019; Dasari & Bernard Tchounwou, 2014).

The formation of reactive oxygen species (ROS) that leads to oxidative stress is also a common mechanism implicated in the cytotoxicity of cisplatin (Brozovic, Ambriović-Ristov, & Osmak, 2010). The formation of hydroxyl radicals and superoxide by cisplatin depends on the concentration of cisplatin and exposure time (Ghosh, 2019). During DNA damage, superoxide anions, a reactive oxygen species (ROS), are produced, leading to apoptosis (Masuda, Tanaka, & Takahama, 1994). Another mechanism of ROS generation by cisplatin occurs through its interactions with thiol groups within proteins. These interactions occasionally form thiyl radicals that interact with molecular oxygen, leading to the formation of ROS (Florea & Büsselberg, 2011). Cisplatin also indirectly increases ROS by binding to free radical scavengers such as glutathione (GSH), reducing number of unbound scavengers available. ROS leads to apoptosis through both intrinsic and extrinsic pathways that include activation of Fas by Fas-ligand, mitochondrial damage, lipid peroxidation, alteration of signal transduction pathways, calcium dysregulation and further DNA damage (Florea & Büsselberg, 2011; Romani, 2022).

In summary, the primary mechanisms of cisplatin are believed to be through the formation of DNA adducts and ROS generation. Although substantial research has been directed towards understanding these mechanisms, critical gaps in knowledge of their origins remain. In particular, some mechanisms have been proposed for the direct generation of ROS as described

above and in section 1.2.5, but other signals responsible for initiating this increase have yet to be fully explored. In the following sections and chapters, I will justify why identifying alternative routes of ROS generation can lead to fruitful discoveries for improving cisplatin usage.

1.2.2 Cisplatin resistance

The development of resistance to cisplatin treatment remains one of the significant limitations for its treatment of solid tumours. Resistance to cisplatin is likely developed through an interaction between decreased drug accumulation, drug metabolism/inhibition, DNA damage repair, and inhibiting pro-apoptotic signalling (Jin et al., 2018). The many mechanisms behind cisplatin resistance are still under investigation, and those reported have been extensively reviewed elsewhere (Amable, 2016; Baird & Gray, 2023; Chen & Chang, 2019; Cocetta, Ragazzi, & Montopoli, 2020; Galluzzi et al., 2012; Galluzzi et al., 2014; Yue, Han, & Zhao, 2023). Herein, we will review a portion of the resistance mechanisms reported to contribute to cisplatin resistance.

Epigenetic and genetic modification by cisplatin initiates many complex changes in protein expression that contribute to its resistance. Epigenetics is defined as modifications to DNA that induce a change in gene regulation without affecting its primary sequence (MedlinePlus, 2023). In the realm of epigenetics, cisplatin treatment has been reported to induce the modification of the methylation patterns within genes, interfere with chromosomal remodelling proteins, modify histone acetylation and methylation, alter the expression of epigenetic reader proteins, microRNAs, long noncoding RNAs, and increase the population of cancer stem cells, which have all been linked to its resistance (Baird & Gray, 2023). These changes often result in protein expression or a lack thereof, that is directly involved in reducing

cisplatin uptake, increasing cisplatin removal, reducing DNA damage, increasing DNA repair, and more.

An increase in drug efflux is a common feature observed within drug resistance. Decreasing the cumulative cisplatin within the cells decreases its ability to interact with DNA and proteins, promoting cancer cell survival. Unfortunately, searching for transporters directly involved in cisplatin efflux has been largely unsuccessful (Shen et al., 2012). Currently, two copper transporters (ATP7A and ATP7B) have been shown to bind and remove cisplatin, reducing its active intracellular concentration (Safaei et al., 2008; Tadini-Buoninsegni et al., 2014). Multiple other proteins involved in the secretion of cisplatin have been implicated in its resistance, but these studies fail to explain how these proteins are involved (Beretta et al., 2010; Shen et al., 2012). A study by Beretta et al. demonstrated that the expression of the efflux proteins multidrug resistance protein 1 and 4 (MRP1; MRP4) increased in cisplatin-resistant cells, but only when the glycosylation of their N-termini were modified (2010). Furthermore, Shen et al. observed a 2.5-fold increase in cisplatin resistance in cells transfected with TMEM205, which is believed to act as a secretion-related protein (2012).

While little evidence has shown MRPs direct involvement in the efflux of cisplatin, they mediate the removal of its conjugates in an ATP-dependent manner (Ishikawa & Ali-Osman, 1993). Aquated cisplatin has a high affinity for cysteine-rich proteins such as GSH and metallothionein proteins, which are known to be excreted from cells by MRP2 and OATs, decreasing intracellular cisplatin concentrations (Amable, 2016; Makovec, 2019). Other transport-related mechanisms responsible for reduced cisplatin accumulation can be attributed to its reduced uptake. Treatment with cisplatin has been shown to trigger the degradation of CTR1, which plays an important role in cisplatin uptake, as described above (Section 1.2.1)(Holzer,

Manorek, & Howell, 2006). Reduced CTR1 leads to decreased cisplatin uptake, therefore facilitating resistance and cell survival.

Increasing DNA repair is another mechanism of cisplatin resistance. The NER system is responsible for removing the majority of cisplatin adducts by removing a section of nucleotides, followed by their resynthesis using the nondamaged DNA strand (Gillet & Schärer, 2006). The overexpression of NER reduces cell sensitivity to cisplatin, while defects in this system increase sensitivity to cisplatin (M. Duan et al., 2020). Preventing apoptotic signalling from DNA lesions by circumventing unrepaired DNA damage is common with resistant cancer cells and can be largely attributed to Y and B family polymerases in a process called translesion synthesis (Rocha et al., 2018). These polymerases lack the ability to proofread and have a broad catalytic site that allows them to pass over DNA lesions. Increased expression of these polymerases is often found in cisplatin-resistant cells (Zhou et al., 2013). Furthermore, their propensity towards inaccurate DNA repair can further facilitate the acquisition of cisplatin-resistant phenotypes (Rocha et al., 2018). Other DNA repair mechanisms, such as the MMR system, can detect cisplatin DNA lesions, but not repair them (Galluzzi et al., 2014). Reduced expression and mutations leading to dysfunction in this system, such as MSH2, MSH6 and MLH1, are often associated with resistance to cisplatin as well (Aebi et al., 1996). Homologous recombination (HR) machinery helps repair double-stranded breaks generated by cisplatin lesions and is often mutated within cancer cells, promoting cisplatin sensitivity (Chen & Chang, 2019; Smith et al., 2010). Cisplatin resistance is often conferred by mutations that allow for the restored function of proteins such as BARCA1/2 in the HR repair system (Shen et al., 2012).

Alterations in pro-apoptotic signal transducers and their related pathways also lead to cisplatin resistance. Pathways commonly affected by cisplatin treatment that confer resistance

are the p53 pathway and MAPK pathways, which, when mutated, reduce cisplatin efficacy in cancer patients (Branch et al., 2000). Mutating the p53 pathway likely confers cisplatin resistance by increasing tolerance of cisplatin adducts, thereby increasing translesion synthesis and gene mutagenesis (Lin & Howell, 2006). Dysfunction in the MAPK pathways facilitates resistance through many mechanisms and has been extensively reviewed by Brozovic and Osmak (2007). Pro-apoptotic proteins involved in cisplatin resistance are BCL-2 family members, c-Jun N-terminal kinase 1 (JNK1), mitogen-activated proteins kinase 14 (MAPK14), and caspases (Galluzzi et al., 2012). Furthermore, it is worth noting that heat shock proteins and upregulation in autophagy show contributions to cisplatin resistance (Ren et al., 2008; Ren et al., 2010; Yamamoto et al., 2001).

These acquired resistance mechanisms are particularly detrimental to cancer treatment because of cancer cells' rapid, uncontrolled growth. They can be rapidly passed on during tumour growth, creating an even larger group of resistant cells prone to further division (Xuan, Haiyun, & Xiaozhuo, 2019). Side effects often arise in response to the toxicity of cisplatin to non-cancerous cells, and these cells often inhibit division following cellular damage, which may even lead to cell death (Eekhout & De Veylder, 2019). Therefore, cells that acquire resistance may not successfully prolong their usage due to the lack of effectively transferring them forward to daughter cells. With that in mind, studying these resistance mechanisms may be beneficial in understanding possible methods to reduce cisplatin toxicity in non-cancerous cells, therefore reducing unwanted side effects. However, the key to mitigating side effects likely lies in reducing the upstream toxicity to non-cancerous cells while maintaining the anticancer efficacy of cisplatin.

1.2.3 Side effects of cisplatin

Treatment with cisplatin has been linked to various toxicities, including gastrointestinal (GI) toxicity, myelosuppression, gonadotoxicity, nephrotoxicity, cardiotoxicity, hepatotoxicity, neurotoxicity, ocular toxicity, and ototoxicity (Romani, 2022). Due to the systemic treatment and non-specific modality of cisplatin, all organs will likely be affected. Unfortunately, the mechanisms behind many of these side effects remain poorly understood, but significant advances have been made over the past few decades. Table 1.1 summarizes the most common clinical side effects of cisplatin and current therapies that have been approved and are either being used in clinical settings or examined for their therapeutic effects (Qi et al., 2019).

Nephrotoxicity is one of the major dose-limiting toxicities of cisplatin and arises due to the kidneys' paramount role in cisplatin excretion. The generation of nephrotoxicity from cisplatin is complex and involves a wide range of processes depicted by Casanova et al. and Qi et al. (Casanova et al., 2021; Qi et al., 2019). Kidney damage from cisplatin presents primarily as tubular damage, which reduces the glomerular filtration rate. The accumulation of cisplatin within the kidneys is often elevated far more than in other organs and is likely related to transporter-mediated uptake of cisplatin. OCTs and CTR1 are primarily expressed within the kidneys and were shown to be directly implicated in cisplatin nephrotoxicity by increasing cisplatin uptake (Ciarimboli et al., 2005; Gorboulev et al., 1997; *World Health Organization: Cancer*, 2023). Cisplatin predominantly damages the proximal and distal tubule cells in the kidneys, leading to cell death (Casanova et al., 2021). The cytotoxic mechanism of cisplatin in kidney cells is through a combination of its regular cytotoxic mechanisms: oxidative stress, DNA damage, inflammation, and the activation of pro-apoptotic pathways (Qi et al., 2019). Oxidative stress likely plays a significant role in nephrotoxicity as the proliferation of tubular epithelial

cells is low under normal conditions (Casanova et al., 2021; Vogetseder et al., 2008; Yuan et al., 2015). Furthermore, the metabolism of cisplatin conjugates within the kidney during cisplatin excretion may generate potent nephrotoxins, leading to tubular cell death (Qi et al., 2019). Some studies have shown that cisplatin can disrupt the ionic homeostasis within tubular cells, which is essential for the proper excretion of waste and water concentrations in urine (Lajer et al., 2005).

One of cisplatin's most common side effects is GI toxicity, which may be linked to the rapid proliferation of epithelial cells within the gastrointestinal tract (Shahid, Farooqui, & Khan, 2018). Nausea and vomiting are reported to occur in roughly 70-80% of patients, often resulting in dose reductions, delays in treatment, or its termination. GI toxicity can be observed directly in the small intestine by examining the damage to villi and microvilli done by cisplatin, which will atrophy even at lower dosages (Shahid, Farooqui, & Khan, 2018). Since microvilli are responsible for increasing the surface area needed to absorb nutrients properly, this would explain the side effects of cisplatin treatment, such as weight loss and anorexia. However, nausea, vomiting, and diarrhea can be attributed to a release of serotonin induced by cisplatin in the GI tract (Cubeddu, 2009; Minami et al., 2003). The serotonin binds to serotonin receptors on the vagal nerve to initiate a vomiting reflex (Minami et al., 2003). The mechanisms behind cisplatin intestinal cell damage are believed to occur through a combination of ROS generation, DNA damage, pro-apoptotic signalling and inflammation within the gut (Shahid, Farooqui, & Khan, 2018).

Neurotoxicity is one of the most severe dose-dependent cisplatin toxicities, often resulting in the cessation of treatment. Most often, cisplatin-induced neuropathy presents as peripheral sensory neuropathy, where the central nervous system either signals abnormally or loses connection to the peripheral nervous system (N. I. o. N. D. a. S. (NIH), 2023; Avan et al.,

2015). Due to the ability of cisplatin to remain within the body years after administration, neuropathy often progresses after treatment is discontinued (Romani, 2022). DNA damage and mitochondrial damage are believed to be the two main mechanisms underlying cisplatin-induced neuropathy (Qi et al., 2019). Cisplatin accumulates within dorsal root ganglia (DRG), which requires a high level of transcription to support their large size and distant connections, which makes them particularly susceptible to DNA damage and apoptosis (Yan et al., 2015). Moreover, cisplatin is known to directly affect the mitochondrial DNA of DRGs, leading to a reduction in ATP production and degeneration of axonal transport (Qi et al., 2019). The most common early symptoms of peripheral neuropathy are numbness, tingling in fingers and toes, and ankle jerks (Avan et al., 2015). In the more extreme cases, neuropathy can lead to Lhermitte's sign, which are electric shock-like sensations that arise after neck flexion (Romani, 2022).

A side effect closely related to neurotoxicity is ototoxicity. Cisplatin-induced ototoxicity (CIO) is a permanent, often bilateral sensorineural hearing loss, most often in the high-frequency range (4000-8000 Hz), but can progress towards lower frequencies in the ordinary conversation range (Chattaraj et al., 2023). The development of CIO is reported to be anywhere in the range of 10-90% of patients and has been observed to occur even after a single dosage of cisplatin (Chirtes & Albu, 2014; Custer, 2022; Knight, Kraemer, & Neuwelt, 2005; McDonald, Mattson, & Hill, 2017; VORUZ et al., 2022). This demonstrates the importance of early detection and proper management to prevent lifelong detriment to patients' quality of life. In most organs, cisplatin is eliminated within days to weeks after injection. However, it remains in the cochlea for months to years after treatment in both mice and humans, which may explain its ability to induce ototoxicity long after treatment ends (Breglio et al., 2017). Unfortunately, the clinical assessment and intervention of ototoxicity are rather poor due to a lack of standardized criteria

and poor adherence to monitoring protocols (Chattaraj et al., 2023; Santucci et al., 2021). Children are at the greatest risk of developing CIO, with the incidence being 22-77% higher in pediatric patients compared to adults (Ghosh, 2019). Although CIO can impact patient quality of life at any age, the consequences of CIO in children are severe. They can impair language development, psychosocial development, and learning difficulties (Knight, Kraemer, & Neuwelt, 2005). Therefore, it is critical to develop pharmacological prevention and mitigation strategies for the development of CIO to improve patient outcomes from cancer treatment with cisplatin. More details on CIO follow in subsequent sections.

1.2.4 Factors associated with increased risk of CIO

Multiple factors have been identified to increase a patient's susceptibility to CIO. As mentioned above, young age is one of the major factors contributing to increased risk of CIO, along with cumulative dosage, old age, diet, poor renal function, melanin content, noise exposure, gender, and genetics (Callejo et al., 2015; Tserga et al., 2019). Understanding these risk factors and their underlying contributions to CIO will allow for a more tailored approach to cancer treatment to reduce the likelihood or progression of side effects. The exact reason as to why age may play a role in susceptibility for CIO is unknown. However, in young children, it may pertain to the fact that the auditory system continues to develop during early childhood and may be more susceptible to damage during development (Meijer et al., 2022).

Cumulative dosage and therapeutic regimen are considered the largest contributing factor to CIO susceptibility. In a retrospective study done using patient data from the St. Louis Children's Hospital, higher dosages per treatment as well as the cumulative dosage have a significant effect on the development of CIO, while the schedule of dosing (number of cycles, total number of doses) had little effect (Camet et al., 2021). Another study found that CIO began

to emerge at dosages above 60mg/m2, which is well within the dosage range for most malignancies (Rademaker-Lakhai et al., 2006). Yancey et al. found that cumulative dosage and male gender have independent effects in children, and in contrast to Camet et al., they also found that patients receiving a single high dosage (100 or 120mg/m2) were at greater risk of developing CIO than those patients that received multiple lower dosages (20 mg/m2 for 5 days) (2012). This difference may be due to the types of cancers being treated, the age of patients, cohort size, etc.

Yancey et al. also found that gender played a significant role in susceptibility, which can be supported by an in vitro study by Huang et al., who determined that cells derived from male patients were more susceptible to cisplatin's effects compared to female-derived cells, but only in one cell line examined (2007; 2012). However, another in vivo study by KirKim et al. contradicted these two studies' findings, which examined cisplatin's effects on male and female Wistar albino rats (2015). They found the hearing of female rates after a single dose of 16mg/kg was significantly worse compared to male rats, however, their sample size was relatively low, with only 7 rats examined in each group. Gender may play a role in CIO susceptibility for various reasons, including drug absorption, drug distribution, metabolism, drug elimination/excretion, hormones, etc. (Soldin & Mattison, 2009). However, its overall contribution is still unclear.

Another factor that has gained substantial interest for contributing to CIO susceptibility is a person's genetics. Several polymorphic genes have been purposed to contribute to cisplatin susceptibility, including those in glutathione-S-transferases (GSTP1, GSTM1, GSTM3), mitochondrial genes, excision repair cross-complementing group 2 (ERCC2), xeroderma pigmentosum complementary group C (XPC), thiopurine S-methyltransferase (TPMT), catechol

O-methyltransferase (COMT), low-density lipoprotein receptor-related protein 2 (LRP2) (megalin), acylphophatase-2 (ACYP2), superoxide dismutase 2 (SOD2), ATP-binding cassette C subfamily member 3 (ABCC3), CTR1, and nuclear factor erythroid 2-related factor 2 (NFE2)-like 2 (NFE2L2) (Callejo et al., 2015; Hagleitner et al., 2015; Rybak et al., 2009; Tserga et al., 2019). The mechanisms behind their contribution to CIO can be found in Tserga et. al., and references therein (2019). However, it is worth noting that one LRP2 polymorphism (rs4668123) was only found to significantly affect CIO when combining multiple studies, demonstrating the importance of sample size when performing studies for genetic associations to disease.

1.2.5 Mechanisms of CIO

The molecular mechanisms behind CIO are still being investigated, but it is generally accepted that cisplatin must enter the cochlea to induce its cytotoxic effects. The blood-labyrinth barrier (BLB) presents as the first obstacle for cisplatin to enter the inner ear, as its regular function is to protect the cochlea from toxins and regulate the passage of fluids and nutrients (Juhn, Rybak, & Prado, 1981; Nyberg et al., 2019). Within the cochlea, cisplatin primarily affects the outer hair cells, cells within the stria vascularis (SV), the organ of Corti, and the spiral ganglion (Sheth et al., 2017). The SV is one of the vascular tissues of the BLB and the first site of inner ear cytotoxicity from cisplatin administration (Figure 1.3)(X. Wang et al., 2023). The SV is principally comprised of two epithelial layers, the basal/intermediate cells and the marginal cells (Liu et al., 2016). The marginal cells express one of the major transporters responsible for cisplatin uptake, CTR1, leading to cisplatin accumulation within the SV and increased permeability of the BLB (X. Wang et al., 2023). SV damage impairs the endolymph's ionic regulation, which is required for normal auditory function (Y. Li et al., 2023). Other routes of cisplatin entry are undoubtedly present within the cochlea but have yet to be determined.

Once cisplatin is taken into the SV, it enters the endolymph, where it can enter the cochlear hair cells via passive diffusion, cation transporters, transient receptor potential (TRP) channels, and mechano-electrical transduction (MET) channels (Y. Li et al., 2023). The mechanisms behind how cisplatin transitions into the endolymph are unknown and require further study. Since the hair cells of the inner ear are senescent, apoptosis from cisplatin DNA adducts that induce cell cycle arrest is unlikely. However, disruption in gene transcription from DNA adducts and/or gene mutations may still play a role, as well as non-DNA damaging mechanisms such as ROS generation, inflammation, mitochondrial dysfunction, and activation of pro-apoptotic pathways (Devarajan et al., 2002).

Currently, it is accepted that an increase in ROS is one of, if not the main cause of CIO (Tang et al., 2021). The different signals believed to initiate ROS generation within cochlear cells include DNA damage, an increase in inflammation and inflammatory signalling, overproduction of oxidases, depletion of antioxidant systems, and dysfunction of the mitochondria and ER (Babolmorad et al., 2021; X. Wang et al., 2023). The antioxidant system in the cochlea includes catalase, superoxide dismutase, reduced glutathione, and glutathione peroxidase (Ravi, Somani, & Rybak, 1995). One major source of ROS generation within the cochlea is NADH oxidase 3 (NOX3), which is highly expressed in the organ of Corti and spiral ganglion (Karasawa & Steyger, 2015). NOX3 is an oxidant that can be over-activated by cisplatin, leading to the generation of ROS. As mentioned in section 1.2.1, ROS can increase directly from cisplatin-induced DNA damage and depletion in antioxidant scavengers such as GSH. The overactivated NOX3 in cochlear cells produces an excess of ROS that can overwhelm endogenous antioxidant systems leaving ROS to induce apoptosis (X. Wang et al., 2023).

However, NOX3 is likely only partially responsible for ROS generation but offers a valuable therapeutic target to help mitigate cell damage.

Inflammation is also considered a significant mechanism of CIO due to the prolonged and exacerbated accumulation of proinflammatory factors and increased ROS generation. Both damage-associated molecular patterns (DAMPs) and cisplatin directly contribute to the inflammation of the cochlea (X. Wang et al., 2023). Toll-like receptors (TLRs) are innate immune signalling molecules, and their functions are discussed in more detail within section 1.4. Toll-like receptor 4 (TLR4) is the most well-studied proinflammatory initiator for its involvement in CIO. After cisplatin damages the cell, TLRs are activated by DAMPs, resulting in the release of inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α), interleukin-1-β (IL-1β), and IL-6 (Behzadi, Garcia-Perdomo, & Karpinski, 2021). Concerning CIO, these cytokines induce inflammation and promote ROS generation (Gong et al., 2020). Moreover, cisplatin has also been shown to bind to TLR4 directly, inducing its activation and leading to proinflammatory cytokine release and subsequent ROS generation (Babolmorad et al., 2021; Domingo et al., 2023).

Increases in ROS also upregulates the expression transient receptor potential vanilloid 1 (TRPV1) channel, which regulates calcium ion influx and can act as a channel through which cisplatin enters cochlear cells, increasing CIO (X. Wang et al., 2023). TRPV1 channels are highly expressed within the basal turn's outer hair cells (OHCs) and are activated by the oxidative stress produced by inflammation and NOX3. This leads to increased cisplatin accumulation as well as increased cytosolic calcium, both of which induce apoptosis in cochlear cells (Mukherjea et al., 2008). Furthermore, the increase in ROS is linked to activation of the

transcription factor signal transducer and activator of transcription 1 (STAT1), which promotes inflammation and activation of p53 leading to cell death (Schmitt, Rubel, & Nathanson, 2009).

Dysfunction of the mitochondria also contributes to ROS generation and CIO. Mitochondrial function to produce energy for the cell and requires tightly regulated ionic gradients. The reactions leading to energy production occur within the inner mitochondrial membrane along the mitochondrial respiratory chain. These complexes perform multiple redox reactions that lead to ATP generation and the conversion of oxygen to water. It is unsurprising that the mitochondria contribute to ROS generation in the presence of cisplatin (Marullo et al., 2013). Cisplatin damages the mitochondria by activating Bcl-2 family members, leading to mitochondrial permeability transition and release of cytochrome C, activating caspases and apoptosis (Devarajan et al., 2002). Cisplatin also binds directly to mitochondrial DNA, inhibiting replication and transcription of genes, leading to the accumulation of misfolded proteins and cell death (Wisnovsky et al., 2013).

Understanding the mechanisms currently known to lead to CIO allows for the investigation into therapies based on rational drug design for treating this debilitating condition. Although the mechanisms behind CIO have yet to be fully elucidated, continued research in this area would uncover new mechanisms and validate new targets for the therapy of CIO. Moreover, understanding how cisplatin induces CIO can improve the design for the next generation of platinum therapeutics and lessen their impact to cells of the inner ear.

1.2.6 Current therapies and management for CIO

Recently, sodium thiosulfate (STS), also known as Pedmark®, was approved by the FDA on September 20th, 2022 as an approved treatment to mitigate CIO ((FDA), 2022). The drug was approved following two clinical randomized controlled trials, SIOPEL6 (NCT006521320 and

COG ACCL0431 (NCT00716976). SIOPEL6 involved 114 patients younger than 18 treated with six cycles of cisplatin and various dosages of STS (10g/m2, 15g/m2, 20g/m2) (Brock et al., 2018). They found that those treated with cisplatin and 20g/m2 STS had a 48% lower incidence of hearing loss and a 6% greater survival rate after three years. Cisplatin was delivered intravenously over 6 hours at 80mg/m2 for two preoperative and two postoperative cycles, while STS was delivered intravenously over 15 minutes six hours after cisplatin infusion. COG ACCL0431 involved 135 pediatric patients being treated with cisplatin with cumulative doses of at least 200mg/m2 or greater ((FDA), 2022; Freyer et al., 2017). Patients treated with STS and cisplatin showed 14% lower incidence of hearing loss compared to cisplatin only controls. Patients receiving STS were dosed at 16g/m2 six hours after cisplatin treatment. Systemically administered STS can complex with cisplatin and be excreted by the kidneys, reducing free cisplatin that may cause undesirable effects (Tang et al., 2021). On top of its improved removal of cisplatin, STS further mitigates CIO by acting as a free radical scavenger, reducing ROS, and preventing its many pro-apoptotic effects. Multiple pre-clinical in vitro and in vivo studies, as well as other clinical studies, have supported the efficacy of STS in reducing CIO, however due to its mechanism of action, there is a possibility that it would reduce cisplatin's anti-tumour effects (Dickey et al., 2005; Duinkerken et al., 2021; Leitao & Blakley, 2003; Muldoon et al., 2000; Neuwelt et al., 1996; Wang et al., 2003). A current solution to this problem is trans-tympanic delivery, which would localize its effects on the inner ear (Duinkerken et al., 2021). Developing a therapy that does not directly interact with cisplatin, but mitigates its side effects could further improve treatment of CIO alongside STS.

Other antioxidants have been examined for their therapeutic efficacy in reducing CIO. These include, but are not limited to, N-acetylcysteine (NAC), amifostine, lipoic acid,

glutathione ester, melatonin, methylthiobenzoic acid (MTBA), and D- or L-methionine, trolox, dexamethasone, resveratrol, ginkgo biloba extract, diethyldithiocarbamate, methylthiobenzoate, caffeic acid, thiourea, ebselen, and salycilate (Fetoni & Astolfi, 2020). Like STS, NAC is a thiol-containing compound that acts as a potent antioxidant and can bind to cisplatin to facilitate its excretion. A randomized nonblinded clinical trial by Yoo et al. evaluated the efficacy of 2% L-NAC and found improved CIO by 18% in two patients, but was overall ineffective (2014). This inconsistent data has been observed with the use of other antioxidants, which has led to their delay in further application for the treatment of CIO (Freyer et al., 2020; Ha et al., 2021). Other promising treatment options are phenolic compounds such as curcumin and ferulic acid, which increase the activation of antioxidant pathways and can downregulate the activity of apoptotic pathways (Fetoni & Astolfi, 2020).

Due to the limited treatment options for CIO, further innovations are paramount to improving the quality of life for patients following cisplatin treatment. Two national associations provide ototoxicity surveillance guidelines: The American Speech-Language-Hearing Association (ASHA) and The American Academy of Audiology (AAA). The ASHA recommends pre-treatment baseline monitoring, interval monitoring during treatment, and post-treatment monitoring at the end of treatment, three months, and six months post-cisplatin therapy (Konrad-Martin et al., 2005). The AAA also recommends pre-treatment baseline monitoring and 3-month post-treatment monitoring but also proposes annual monitoring in those that have undergone or are currently undergoing head and neck radiation as this is a factor for increased risk of CIO ("American Academy of Audiology Position Statement and Clinical Practice Guidelines: Ototoxicity Monitoring," 2009). Audiological testing to examine cochlear injury includes basic audiological assessment, otoacoustic emission measurement, extended high

frequency (EHF) pure tone audiometry, speech and impedance audiometry, auditory brainstem response test, and distortion product otoacoustic emissions (DPOAEs) testing (Chirtes & Albu, 2014). These tests evaluate the ability of a patient to hear, especially in the high frequencies (4-8 kHz) often affected by CIO. DPOAEs are particularly important in testing for CIO as it is more sensitive than EHF testing, depending on the integrity of hair cells themselves, and does not rely on patient response, which could be compromised or complicated, especially in children, during and after their treatment (Chirtes & Albu, 2014).

In summary, cancer treatment is often considered a double-edged sword in that it helps or can even cure a life-threatening disease but can also leave you with lifelong conditions impacting your everyday life. While there are attempts to reduce these side effects, many have been unsuccessful due to a variety of challenges. One challenge arises in the availability of appropriate platforms that are accessible yet faithfully represent the in vivo complexity for dissecting the mechanisms behind CIO. Furthermore, many of these platforms fall short in their ability to empirically test innovative anti-CIO approaches. As such, in the following section, we consider a role for zebrafish larvae in assisting with filling this gap.

1.3 Zebrafish as a model of cancer and CIO

1.3.1 Zebrafish as a model organism

The use of zebrafish as an animal model has grown considerably in past decades and has been used to study human nervous system diseases, cardiovascular and metabolic diseases, mental disorders, cancer and more (Choi et al., 2021; Philip et al., 2017; Tonon & Grassi, 2023; K. Wang et al., 2023). The utility of in vivo models compared to cell cultures stems first and foremost from the benefit of examining the physiological functions and systemic interactions that give rise to a response within an organism (Barré-Sinoussi & Montagutelli, 2015; Murphy, 1991;

World Health Organization: Cancer, 2023). For example, pepsin secretion requires inputs from the nervous system and other hormones such as secretin, gastrin, and cholecystokinin (Murphy, 1991). Using whole organisms also allows for a broader range of disease phenotypes to manifest during experimentation (MacRae & Peterson, 2015). Cell culture can facilitate the formation of hypotheses, specific interactions at a cellular level, and postulation of models, but whole organisms are needed to demonstrate their application. The use of zebrafish as an animal model for disease and novel therapeutics was proposed in 1981 by Streisinger et al., who examined the production of genetic homozygotes in zebrafish embryos (1981). Later in 2001, the zebrafish genome-sequencing project established a complete genome sequence for zebrafish, which led to the finding that greater than 80% of disease-related genes in humans can be related to at least one zebrafish ortholog (Howe et al., 2013). The sequencing and mapping of the zebrafish genome greatly facilitated its transition into the spotlight as an animal model.

Several innate characteristics of the zebrafish are advantageous over rodents and other models when studying vertebrate diseases (Adefegha et al., 2022; Hason & Bartůněk, 2019; Kwiatkowska et al., 2022; Raby et al., 2020; Tonon & Grassi, 2023). They have large clutch sizes (>100 embryos) that develop outside the body quickly, which allows for high throughput assays and large studies to be performed in a relatively short period (Philip et al., 2017). Compared to murine models, which often only have single-digit sample sizes, the large number of zebrafish larvae also facilitates simultaneous testing of multiple hypotheses, or empirical testing of interventions, at once. Multiple different technologies and assays have been developed to facilitate larval phenotyping and observation to optimize the utilization of their high numbers (d'Alençon et al., 2010; Lessman, 2011; McCarroll et al., 2016; Miscevic, Rotstein, & Wen, 2012; Pulak, 2016; G. Wang et al., 2015; Yew et al., 2012). Not only can they produce mass

number of embryos at once, but their larvae are also small (~4 mm in length) and robust, which helps reduce housing and maintenance costs compared to other models. Furthermore, the small size of the animals means they will often require less reagent when testing, allowing for the conservation of materials with greater statistical output. Moreover, the transparent larvae make it possible to visualize and document processes happening within the body in real-time, which is more difficult in rodents. Together, these advantages demonstrate the added value zebrafish provide as an animal model that complements what can be traditionally achieved within cell culture and more established models such as rodents.

As with every model, there are limitations and drawbacks to zebrafish, many of which parallel its advantages. The ability to examine interactions between a whole system is an advantage but can also make it difficult to isolate and identify underlying causes of an observed effect. Furthermore, the small size of zebrafish can make it challenging to collect and analyze tissue. Zebrafish are also aquatic poikilotherms, and their environment can both complicate and facilitate drug administration and functional assays. Their inability to consistently regulate body temperature can also be a challenge during xenotransplantation due to compromising the cells from humans, which require roughly 37°C temperatures to survive (Barriuso, Nagaraju, & Hurlstone, 2015). Furthermore, relatively few antibodies are available for immunohistochemical studies compared to other animal models. Lastly, their time to sexual maturity is around three months, which is similar to rodents, and provides no additional benefit to the generation of knockouts and transgenics (Astone et al., 2017; Philip et al., 2017).

1.3.2 Zebrafish in cancer research

As described above, there are multiple advantages to using zebrafish as a model for vertebrate disease and recently, zebrafish contributions to cancer research have grown rapidly.

Over 50 models of human cancer have been established in zebrafish that closely resemble their human counterparts (Casey & Stewart, 2020). Some of these cancers include leukemia, melanoma, pancreatic cancer, liver cancer, testicular cancer, glioma, thyroid cancer, neuroblastoma, and sarcoma (Astone et al., 2017; Casey & Stewart, 2020; White, Rose, & Zon, 2013; Yen, White, & Stemple, 2014). Due to the high degree of protein and signalling conservation within zebrafish, these cancer models have helped us identify pathways that underlie their growth, metastasis and treatment response (Yen, White, & Stemple, 2014). More importantly, a plethora of new therapies and drugs have been identified that mitigate disease phenotypes and have been moved into clinical trials (Letrado et al., 2018). Zebrafish have also been paramount in understanding the molecular actions of these drugs and appropriate dosages. Some of these drugs include ProHema for the treatment of hematological malignancies, Rosuvastatin as an antiangiogenesis drug, leflunomide for melanoma, amitriptyline and paroxetine for the treatment of hepatocellular carcinoma, and rapamycin, disulfiram, and tanshinone for treatment of melanoma (Letrado et al., 2018). Detailed reviews on specific cancer models generated in zebrafish can be found elsewhere (Astone et al., 2017; McConnell, Noonan, & Zon, 2021; Yen, White, & Stemple, 2014), herein a brief outline of techniques used to generate cancer models are discussed.

In cancer research, zebrafish provide a unique advantage over rodent models because of their reduced cost and time for assessing novel therapies, genetic and drug screening scalability, and optical transparency for monitoring and in vivo imaging (Casey & Stewart, 2020). However, rodents remain a widely implemented pre-clinical model due to their physiological similarity to humans, accurate drug delivery, dosing and metabolism (Singhal et al., 2023). Several strategies have been used to generate zebrafish cancer models, primarily engineering mutant lines,

generating transgenic lines and xenotransplantation (Letrado et al., 2018). Forward genetic screens using carcinogens and retroviruses in zebrafish are often used to identify new genes responsible for generating cancers and their mechanism of action (Kwiatkowska et al., 2022; Raby et al., 2020). N-ethyl-N-nitrosourea (ENU) is a commonly used reagent to induce point mutations in Drosophila, mice, and zebrafish (Grunwald & Streisinger, 1992; Mullins et al., 1994; Russell et al., 1979; Zimmering & Thompson, 1984). ENU is a highly potent mutagen that induces mutations in pre- and post-meiotic cells, making it useful for screening the effects of new mutations (Trevarrow, 2011). Following the generation of tumours, molecular techniques such as oligonucleotide microarrays and in situ hybridization are used to determine gene expression profiles, allowing for the identification of mutations that give rise to these malignancies and their similarities to human tumour expression profiles (Koudijs et al., 2005; Lam et al., 2006; Sonawane et al., 2005). Reverse genetic screens through targeting induced local lesions in genomes (TILLING) is another commonly used technique to generate zebrafish cancer models that are known to be linked to specific genes (McCallum et al., 2000). This approach can generate mutations in almost any allele within zebrafish and produce specific malignancies for further study (Wienholds et al., 2002).

While classic reverse genetic approaches are highly useful, and TILLING holds some promise, other forward genetic manipulation techniques have since been developed. Gene editing technologies such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), were innovative but are now supplanted mainly by clustered regularly interspaced short palindromic repeats (CRISPR). These have improved our ability to precisely modify and study genes, especially those involved in cancer generation, progression, and prevention. These site-specific endonucleases generally work by providing a protein (zinc-finger, TALE, Cas9,

respectively) with instructions (either through its structure (ZFN and TALEN) or an exogenous sequence (CRISPR)) that allows them to recognize a portion of the genome to induce single or double-stranded breaks leading to mutations during the repair process (Gaj, Gersbach, & Barbas, 2013). On the other hand, transgenic zebrafish models allow for the expression of genes throughout or within specific tissues of the zebrafish to determine their effects (Raby et al., 2020). Using a tissue-specific promoters, different oncogenes such as *Myc*, *Akt2, mitfa*, *ptf1a*, and more have been expressed to give rise to models of acute lymphoid leukemia, different types of sarcomas, melanoma, pancreatic cancer, liver cancer, brain tumours, etc. within their respective tissues (Borga et al., 2019; Gutierrez et al., 2011; Patton et al., 2005; Yang et al., 2004). Moreover, combining the optical advantages of the zebrafish and transgenics facilitates the use of fluorescent proteins that allow for the visualization of cells, processes, and subcellular structures involved in cancer (Gamble et al., 2021).

Another advantageous model of human cancers gaining substantial notoriety in zebrafish is xenograft transplantation. Xenograft transplantation is the process of taking living cells from one species and implanting them into an organism of another species (Hason & Bartůněk, 2019). Xenotransplantation in other animal models, such as rodents, is limited by the number of animals that can be grafted, the rejection of grafted cells by an immune response, and the inability to view the tumour and its cells in vitro. Zebrafish do not fully develop an adaptive immune response until roughly 28 days post-fertilization (dpf), allowing for the xenotransplantation of human cells without immune rejection, suppressive drugs, or immunocompromised animal models (Lam et al., 2004). This makes zebrafish embryos and larvae the preferred age for xenotransplantation, with two dpf embryos being the most commonly used timepoint (Gamble et al., 2021). Due to their fast development, larvae already have brains, hearts, and livers. The use

of xenografts in zebrafish is especially beneficial when examining therapeutics and processes such as angiogenesis, tumour cell extravasation, migration, and metastasis due to the ability to visualize these processes in vivo (Letrado et al., 2018; Nicoli et al., 2007). Unfortunately, zebrafish larvae grow and flourish in 28°C, but human cells are only stable within a narrow range surrounding 37°C (Gamble et al., 2021). However, zebrafish can survive in temperatures ranging between 32°C and 36°C and others have reported 34-35°C as a compromise for both the cells and the zebrafish host (Barriuso, Nagaraju, & Hurlstone, 2015; Singhal et al., 2023). Another advantage in zebrafish comes from their small size, as only a population of 100 donor cells or less is often required for transplantation, while much greater cell masses are required in rodent models (Gamble et al., 2021). This also comes with the disadvantage that small cell populations can increase heterogeneity between xenotransplant models. In light of these advantages, the major utility of zebrafish xenografts comes from their ability to evaluate anti-tumour therapies and the impact of other drugs co-administered alongside them. The xenograft transplantation models of human cancers in zebrafish and their drawbacks have been discussed previously in more detail elsewhere (Barriuso, Nagaraju, & Hurlstone, 2015; Cabezas-Sáinz et al., 2020; Hason & Bartůněk, 2019).

1.3.3 Drug discovery in zebrafish

One of the most prominent applications of the models described above is in discovering novel therapeutics for disease. Zebrafish can readily identify compounds that mitigate or cure disease phenotypes, characterize the compounds used, and observe any additional benefits or issues with drug application. Letrado et al. reported that up until 2018, there were 355 instances of zebrafish being used for drug discovery in cancer therapy (2018). Compared to screening drugs in cell culture based on their action on a specific target, phenotypic drug screening in

zebrafish is beneficial because it does not require a known target to be validated to determine its effects (MacRae & Peterson, 2015; Rennekamp & Peterson, 2015). One can observe effects generated through multiple molecular targets simultaneously. For example, the effects of ezetimibe were known before Niemann-Pick C1-like protein 1 (NPC1L1) was discovered as its molecular target, and the antiarrhythmic agent amiodarone was found to exert its effects on multiple structures at once long after its discovery (Clader, 2004; Kodama, Kamiya, & Toyama, 1997). Zebrafish also provide early insight into the toxicity of novel drugs, which is often not performed until late into preclinical testing in rodent models (MacRae & Peterson, 2015). This allows drugs to be removed from further testing earlier in development, conserving time, effort, and money. Zebrafish are often used to examine novel classes of compounds, but because of their ability to demonstrate multiple effects at once, they have also been used to discover new uses and targets for existing compounds. For example, cyclooxygenase (COX) inhibitors that were already clinically approved as nonsteroidal anti-inflammatory drugs, were identified as potential therapeutics for leukemia in a transgenic acute myeloid leukemia zebrafish model through a high throughput drug screen (Yeh et al., 2009).

One hurdle to overcome when testing drugs on zebrafish is determining the mechanism of action after observing phenotypic effects. For example, direct binding partners are often more easily identified within cell culture. This shortcoming can be overcome by comparing the structure of the novel compound with other well-established compounds, examining its binding activity through affinity purification, looking for compounds that produce similar phenotypes, assessing expression differences within the model, computational analyses, protein microarrays, and more (Rennekamp & Peterson, 2015; Tamplin et al., 2012; Ziegler et al., 2013).

Screening of compounds in zebrafish is most often performed through bath application of the drugs. Bath application implies that the compounds are administered to the media within which the zebrafish are immersed. They can then absorb compounds through their skin and by swallowing (Zhang et al., 2015). However, this can be complicated depending on the characteristics of the drug, especially if it has a low solubility, permeability to the zebrafish, or is insoluble in water. These issues can be overcome by first dissolving the compound of interest in a vehicle, such as DMF (Dimethylformamide) or DMSO (Dimethyl sulfoxide), which can then be added to the media at a known concentration. Another option is through direct administration of the drug, but this reduces the high-throughput nature of the model and is much more invasive, possibly interfering with experimental outcomes (Kinkel et al., 2010).

1.3.4 The zebrafish acoustico-lateralis system and CIO

CIO primarily affects the inner ear hair cells within mammals. Zebrafish do not contain a cochlear structure; however, they do contain multiple structures homologous to the inner ear's hair cells of mammals. This subsection will describe why their acoustico-lateralis system provides an excellent model for studying CIO and related therapies.

Like all fish, zebrafish likely use their auditory system to learn about their environment, protect against threats, and communicate with one another (Popper & Sisneros, 2022; Poulsen et al., 2021). The acoustico-lateralis system of zebrafish is functioning by five dpf and refers to their inner ear and lateral line system (Bhandiwad et al., 2018; Han et al., 2020; Lara et al., 2022; Moorman, 2001). Unlike mammals, zebrafish do not possess a cochlea within their inner ear, however, they have an accessory hearing structure called the Weberian ossicle that transmits vibrations from their swim bladder to the inner ear (Ladich & Schulz-Mirbach, 2016; Moorman, 2001). It is important to note, however, that the Weberian ossicle is not developed in zebrafish

larvae, although they are reported to process auditory information as well as adults (Higgs et al., 2003; Inoue, Tanimoto, & Oda, 2013). In addition to their Weberian ossicle, the zebrafish inner ear is composed of three semicircular canals, the saccule/saccule otolith, lagena/lagena otolith, sinus impar, utricle/utricle otolith, macula lagenae, macula sacculi, and macula utriculi (Baxendale & Whitfield, 2016; Ladich & Schulz-Mirbach, 2016; Popper & Sisneros, 2022). The otoliths begin to form around 18 hours post fertilization (hpf) and utricular and saccular otoliths are fully formed by 36 hpf (Moorman, 2001).

The inner ear can be divided into upper and lower sections, with the upper inner ear comprising the vestibular system and consisting of the semicircular canals and the utricle (Ladich & Schulz-Mirbach, 2016). The lower inner ear consists of the saccule and lagena. The maculae are embedded within their respective otolith and comprise sensory hair cells. Like other fish, the vestibular system is used to help determine body position and motion by changes in acceleration, while the saccule and lagena are the main auditory organs (Inoue, Tanimoto, & Oda, 2013; Ladich & Schulz-Mirbach, 2016). Adult zebrafish can detect frequencies up to 4000 Hz, which is mainly mediated by their Weberian ossicle (Poulsen et al., 2021). Larvae, on the other hand, are reported to respond behaviourally to frequencies from 50Hz to 1200Hz and similar to goldfish, they have been shown to respond maximally with frequencies between 100-400Hz (Bang et al., 2002; Bhandiwad et al., 2018; Lu & DeSmidt, 2013; Zeddies & Fay, 2005). These studies use a wide range of methods to examine larval “hearing”, including recording microphonic potentials with direct stimulation to the inner ear (Bang et al., 2002), measuring the acoustic evoked startle response (Bhandiwad et al., 2018; Lu & DeSmidt, 2013), measurement of the auditory brainstem response (Higgs et al., 2003), and examining neuronal activation using calcium imaging (Poulsen et al., 2021; Privat et al., 2019; Vanwalleghem, Heap, & Scott, 2017).

The lateral line is reported to be most sensitive to lower frequencies between 50 and 450 Hz, with a loss in auditory responsiveness occurring with a reduction in hair cells (Bang et al., 2002; Lara et al., 2022; Privat et al., 2019). This begins to demonstrate the potential of zebrafish larvae for modelling hearing loss due to hair cell damage.

The zebrafish lateral line is used to detect changes in water movement, allowing them to orient themselves in currents, detect obstacles, prey, predators, and each other (Colombi, Scianna, & Preziosi, 2020; Thomas et al., 2015). The lateral line is comprised of superficial clusters of mechanosensory hair cells called neuromasts, which are deposited around the head in the anterior lateral line (aLL) system and the body in the posterior lateral line (pLL) system (Ghysen & Dambly-Chaudière, 2004; Hardy et al., 2021). The development of the pLL begins around 20 hpf, with the formation of a placode of ~100 cells called the primordium, which is posterior to the otic vesicle of the inner ear (Colombi, Scianna, & Preziosi, 2020; Dalle Nogare & Chitnis, 2017; Gompel et al., 2001). The primordium migrates using signals from chemokine stromal-derived factor 1 (SDF1) down the body of the larvae and deposits 6-8 neuromast progenitors (proneuromasts) in stereotypical positions (Colombi, Scianna, & Preziosi, 2020; Ghysen & Dambly-Chaudière, 2004; Valentin, Haas, & Gilmour, 2007). The deposition and development of proneuromasts continues until around 48 hpf, by which time they have differentiated into support cells and hair cells to form mature neuromasts (Colombi, Scianna, & Preziosi, 2020; Gompel et al., 2001). pLL neuromasts are connected to the hindbrain via peripheral bipolar neurons that merge into a cluster called the pLL ganglion behind the ear (Alexandre & Ghysen, 1999). The lateral line becomes fully functional by five dpf and continues to develop throughout adulthood (Domarecka et al., 2020; Ma & Raible, 2009; Thomas et al.,

2015). Due to the highly regulated developmental process of the lateral line, the positions and structure of neuromasts between different larvae are conserved (Gompel et al., 2001).

Neuromasts are comprised of a ring of support cells surrounded by a central cluster of 8-20 hair cells at five dpf (Ma & Raible, 2009). While there are differences, the hair cells within neuromasts share functional and morphological similarities to the inner ear hair cells of mammals (Chiu et al., 2008; Domarecka et al., 2020). Similarities are even found at the genetic level, with many genes implicated in mechanotransduction, hair cell integrity, synapse transmission, and survival, sharing a close counterpart in mammals (Nicolson, 2005). Neuromast hair cells contain stereocilia and kinocilia organized into rows that increase in height towards one end (Nicolson, 2005). The hair cells in neuromasts are organized into two groups, with their cilia bundles mirroring one another, which allows them to respond to stimuli from opposite directions (Hardy et al., 2021; Thomas et al., 2015). The cilia are surrounded by a gelatinous cupula that deforms during movement, activating hair cells (Nicolson, 2005). Along with the high degree of similarity to mammals, the superficial position of the lateral line neuromasts are accessible to drug treatment, making them an ideal model for studying mechanisms of hair cell death from ototoxic insult.

Zebrafish larvae are a common model used to investigate ototoxicity and compounds capable of protecting against it (Domarecka et al., 2020). Fluorescent microscopy involving the lateral line is a well-established method for examining ototoxicity (Baxendale & Whitfield, 2016; Lee et al., 2022; Ou, Raible, & Rubel, 2007; Wertman et al., 2020). Dyes such as FM1-43, DASPEI (2-(4-(Dimethylamino)styryl)-N-ethylpyridinium iodide), and YO-PRO1 are all commonly employed for these purposes (Baxendale & Whitfield, 2016; Owens et al., 2008). Stains are added to the media and absorbed by the hair cells after cisplatin treatment through bath

application. Since the compounds mentioned above are vital dyes, this method allows for the selective examination of living hair cells and support cells within neuromasts, as they are the only exposed structures available for labelling (Baxendale & Whitfield, 2016). DASPEI is a mitochondrial stain capable of depicting cellular survival based on mitochondria function and density (Wong et al., 2023). Since cisplatin induces neuromast hair cell death in a dose dependent manner, DASPEI fluorescence can be used to assess hair cell viability based on fluorescent intensity (Harris et al., 2003; Ton & Parng, 2005).

Another method that complements morphological analyses for examining ototoxicity is using behavioural assays. Due to the requirement of functional hair cells in certain behaviours, ototoxic compounds have been shown to impair the startle response and rheotaxis (Buck et al., 2012; Niihori et al., 2015; Todd et al., 2017). The startle response is an autonomous reflex evoked during exposure to sudden aversive external stimuli and is present within larvae by five dpf, aligning with the maturation of the lateral line neuromasts (Buck et al., 2012). Behavioural analysis provides information on the ability of hair cells to function after exposure to ototoxic substances and compounds purposed as otoprotectants. Furthermore, with advancements in technology available, high throughput behaviour assays are emerging in zebrafish movement tracking, allowing for the screening of thousands of chemicals and their effects (Bang et al., 2002; Han et al., 2020; Tantry, Harini, & Santhakumar, 2022).

While the lateral line provides unique advantages to examining ototoxic compounds, it also has several limitations. First, it does not adequately model cisplatin's barriers to entering and inducing its toxic effects on hair cells. Second, many structures primarily affected by cisplatin, such as the SV, are absent in zebrafish. This prevents any information on the effects of drugs on these structures from being observed. Third, zebrafish share only 70% homology with the human

genome and thus may lack or express proteins not found in their mammalian counterparts. Additionally, zebrafish can regenerate hair cells and associated structures, which does not occur in terminally differentiated mammalian hair cells (Hardy et al., 2021). It is worth noting that it takes up to 24 hours for the functional regeneration of hair cells after damage. Therefore, a mammalian system may not be faithfully represented by zebrafish and would require complementing studies in mammalian models to confirm findings.

1.4 The innate immune system and toll-like receptor 4

1.4.1 The innate immune system

Innate immunity is the first system to evolve as a line of defense against invading pathogens and foreign particles (Li & Wu, 2021). It plays a crucial role in generating an inflammatory response to eliminate pathogens while priming and activating the adaptive immune system (Kaur & Secord, 2019). The skin acts as a physical barrier between the environment and the host, protecting it from many possible threats, along with mucosal tissue, low stomach pH, saliva, and other secretions (Riera Romo, Pérez-Martínez, & Castillo Ferrer, 2016; Turvey & Broide, 2010). If a pathogen can enter the host, it will be greeted with a barrage of circulating innate immune proteins (Turvey & Broide, 2010). These include complement proteins, LPS binding protein (LBP), pentraxins, antimicrobial peptides, and collectins. These proteins help initiate immune responses and clearance of pathogens (Turvey & Broide, 2010). The other major component of innate immunity is through a cellular defence system and includes monocytes, neutrophils, macrophages, dendritic cells, natural killer cells, mast cells, eosinophils, and basophils (Li & Wu, 2021). These cells mainly use three strategies in the recognition of foreign invaders: recognition of DAMPs, recognition of cells lacking expression of host-specific molecules, and recognition of pathogen-associated molecular patterns (PAMPs) (Kaur & Secord,

2019). PAMPs are essential in a microorganism function, are often found in multiple different pathogens, and have unique characteristics from the host that enables a distinction between host and invasive microbes (Zindel & Kubes, 2020).

PAMP recognition is achieved by displaying germ line-encoded receptors on their surface called pattern recognition receptors (PRRs) (Brubaker et al., 2015). PRRs are categorized into five classes based on domain homology: AIM2-like receptors (ALRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene-1-like receptors (RLRs), and nucleotide oligomerization domain-like receptors (NLRs), toll-like receptors (TLRs). NLRs are composed of a nucleotide-binding domain, a leucine-rich repeat domain, and an N-terminal effector domain. They are responsible for recognizing intracellular PAMPs such as diaminopimelic acid, ssRNA, muramyl dipeptide (Li & Wu, 2021). RLRs are also intracellular receptors mainly responsible for the recognition of viral nucleic acids (Rehwinkel & Gack, 2020). They are classified through the central DexD/H helicase domain that acts as both an ATPase and helicase. The last intracellular class of PRRs are ALRs, which can recognize intracellular DNA through their C-terminal HIN-200 domain and initiate inflammasome formation through their N-terminal domain (Man, Karki, & Kanneganti, 2016). CLRs are phagocytic transmembrane and secretory receptors that recognize carbohydrate PAMPs through their carbohydrate recognition domain (Dambuza & Brown, 2015). Finally, TLRs are transmembrane signalling proteins that function to bind a wide range of PAMPs depending on their location and structure (Kawai & Akira, 2010). TLRs will be discussed in more detail in later sections. The activation of PRRs leads to both transcriptional and cellular processes, including the release of proinflammatory cytokines and interferons, phagocytosis, autophagy, apoptosis, and cytokine processing (Li & Wu, 2021).

1.4.2 Fish vs. amniote innate immune system

The innate immune system is well conserved across vertebrate groups due to the importance of the innate immune system and its components for initiating defences against pathogens (Riera Romo, Pérez-Martínez, & Castillo Ferrer, 2016). Fish rely heavily on their innate immune system mainly due to their adaptive immune response being less efficient than mammals (Kordon, Karsi, & Pinchuk, 2018). The physical barriers of fish and other vertebrates maintain the function of protecting from environmental factors and can express many shared antimicrobial peptides (Rakers et al., 2013). Unlike many amniotes, fish epithelial cells excrete mucus containing antimicrobial substances to act as a sealant and protect them from pathogens in their aquatic environment. The mucus that fish excrete on their skin contains conserved innate immune proteins, with some teleost fish expressing homologs of pentraxins, lectins, lysozymes, antibacterial peptides, and immunoglobulin M (Uribe et al., 2011; Vasta, Ahmed, & Odom, 2004). Lin et al. examined the acute phase response within zebrafish, finding many similarities to mammals as well as some fish specific acute phase proteins such as LECT2 (2007). Several complement proteins have also been described in teleost fish, such as C5a, an important chemokine in macrophage and neutrophil recruitment, and C3, which promotes phagocytosis of bacteria during infection (Dalmo & Bøgwald, 2022; Uribe et al., 2011).

Regarding innate immune cells, fish produce macrophages, natural killer-like cells, neutrophils, eosinophils, basophils, monocytes, and dendritic cells (DCs). In terms of innate immune PRRs, they also express multiple TLRs, CLRs, RLRs and NLRs (Chang, 2021; Dalmo & Bøgwald, 2022; Li et al., 2017; Mokhtar et al., 2023; Sahoo, 2020; Subi & Shabanamol, 2022; L. Zhang et al., 2018). ALRs are the one class of PRR not conserved within fish, and their absence suggests that fish have another mechanism for coping with pathogenic intracellular

DNA (Li et al., 2017). However, it is worth noting that Boudinot et al. have reported the presence of two HIN200 domain containing genes in coelacanths (Boudinot et al., 2014). Teleost fish have two natural killer (NK) cell homologs, non-specific cytotoxic cells and NK-like cells, but these cell types are highly variable in their morphology and activities (Mokhtar et al., 2023; Yoshida et al., 1995). Macrophages are well documented within zebrafish and other teleosts, and play a crucial role in adaptive immune cell activation and phagocytosis of foreign substances (Dalmo & Bøgwald, 2022). Moreover, macrophages express TLRs on their surfaces and are an essential source of chemokines, ROS and proinflammatory cytokines such as TNF, IL-1, and IL-6, which are used to activate immune responses (Arango Duque & Descoteaux, 2014). DCs also express TLRs and mainly function in activating T-lymphocytes as APCs (Kordon, Karsi, & Pinchuk, 2018). DCs have been characterized in several teleost fish, including zebrafish (Alesci et al., 2022; Lugo-Villarino et al., 2010). Granulocytes, including neutrophils, basophils, and eosinophils, play essential roles in inflammation. Depending on the pathogen, in zebrafish these cells degranulate and excrete histamines, chemokines, proteases, and ROS at infection sites to activate tissue-resident immune cells and recruit leukocytes that further increase inflammation (Campos-Sánchez & Esteban, 2021).

1.4.3 Vertebrate toll-like receptors

As mentioned in previous sections, the transparency, genetic similarity to humans, high-throughput nature, the plethora of molecular tools available, and the ability to independently study innate and adaptive immunity all contribute to the use of zebrafish as an excellent model for studying innate immunity (Gomes & Mostowy, 2020; Martinez-Navarro et al., 2020; Rosowski, 2020; van der Sar et al., 2004). The innate immune system in zebrafish is active as early as 28 hours post-fertilization, expressing macrophage cells capable of distinguishing

between self and foreign bodies, performing phagocytic behaviour, and mounting immune responses to bacterial infections (Herbomel, Thisse, & Thisse, 1999; Rosowski, 2020). Following macrophages, neutrophils are expressed by two days post-fertilization, representing the two largest immune cell populations within larval zebrafish (Le Guyader et al., 2008; Renshaw & Trede, 2012). Other innate and adaptive immune components such as NK cells, DCs, eosinophils, the complement system, cytokines, toll-like receptors (TLRs), nucleotide-binding oligomerization (NOD)-like receptors (NLRs), T-cell receptors (TCRs), lymphocytes, immunoglobulins, major histocompatibility complexes and more have been identified in zebrafish (Renshaw & Trede, 2012; Rosowski, 2020; van der Sar et al., 2004). As in mammals, the first immune cells to arrive at the site of infection or tissue damage are neutrophils, which are attracted by an increase in hydrogen peroxide created by dual oxidase (Martinez-Navarro et al., 2020; Renshaw & Trede, 2012). Other molecules released during injury can activate PRRs, such as TLRs and NLRs.

Like many other organisms, zebrafish innate immune cells use PRRs for detection of pathogen associated molecular patterns (PAMPs). Specifically, TLRs recognize PAMPs, which are components shared between pathogens, but not host cells. This allows for a high level of evolutionary conservation in TLRs, as changes in the specificity of these receptors may be disadvantageous (Carlos G.P. Voogdt, 2016). TLRs are ancient PRRs that arose >700 million years ago, originally containing only a transmembrane domain with a cytosolic Toll/IL-1 receptor (TIR) domain (Behzadi, Garcia-Perdomo, & Karpinski, 2021). TLRs are widely expressed throughout an organism but play a significant role in antigen presenting cells (APCs) such as macrophages, dendritic cells, and B Cells (Roach et al., 2013; van der Vaart, Spaink, & Meijer, 2012). In a recent study by Liu et al., vertebrate TLRs were grouped into eight

subfamilies based on their sequence homology and ligand recognition (Carlos G.P. Voogdt, 2016; Liu et al., 2020; Quiniou, Boudinot, & Bengtén, 2013; Wang et al., 2016). The function and presence of all TLRs is not ubiquitous between vertebrates, but in general, the TLR1 family consists of TLR1, 2, 6, 10, 14, 18, 25 and 27 and recognizes lipids and lipoproteins. The TLR3 family recognizes double-stranded RNA (dsRNA), while the TLR4 and TLR5 family recognize LPS and bacterial flagellin, respectively. The TLR7 family consists of TLR7-9 and recognizes nucleic acid motifs in ssRNA and CpG-containing DNA. The TLR11 family consists of TLR11, 12, 19 and 20, with TLR11 and 12 recognizing profilin (Koblansky et al., 2013), TLR19 recognizes dsRNA (Liao & Su, 2021), and TLR20 lacks a known ligand. Like the TLR3 and TLR7 subfamilies, the TLR13 subfamily consisting of TLR13, 21, 22 and 23 also recognizes nucleic acid motifs such as CpG-containing DNA, dsRNA, and ssRNA (Liao & Su, 2021). TLR15 subfamily is only found in avian and reptile genomes and was found to be activated in a unique fashion where microbial proteases cleave the ectodomain (De Zoete et al., 2011; Liu et al., 2020; Wang et al., 2016).

The number of TLRs an organism encodes varies, with humans and mice encoding 10 and 13 TLRs, respectively (Fitzgerald & Kagan, 2020). Drosophilae encode nine toll receptors, birds encode 10 TLRs, reptiles encode 13 TLRs, amphibians encode 14 TLRs, and the purple sea urchin encodes 222 TLRs (K. L. Wang et al., 2021). The reason for such a range in TLRs encoded by an organism unknown, but it is speculated to be due to a variety of factors, for example the evolution of an adaptive immune system, the environmental pathogens an organism encounters, evolutionary pressures, and gene duplication events (Carlos G.P. Voogdt, 2016; Glasauer & Neuhauss, 2014; Hughes & Piontkivska, 2008; Ishii et al., 2007; K. L. Wang et al., 2021). Zebrafish encode 20 Tlr homologs, including Tlr1-5, Tlr7-9, and Tlr18-22 (H. Chen et al.,

2021). Zebrafish Tlr1-3, Tlr5, and Tlr7-9 are orthologs to mammalian TLRs, having similar functions and structures. Others, such as Tlr18-22, represent fish-specific TLRs and appear to have little to no connection with the mammalian genome (Behzadi, Garcia-Perdomo, & Karpinski, 2021; K. L. Wang et al., 2021). However, some TLR genes, such as Tlr4, have high sequence similarity to their mammalian counterpart, hence their name and grouping with other TLR4s, but their function is still under scrutiny within the literature (Loes et al., 2021; Sepulcre et al., 2009; Sullivan et al., 2009).

Zebrafish Tlr4, 5, 8, and 20 have duplicate genes, which is likely due to fish undergoing whole genome and tandem duplication events (Glasauer & Neuhauss, 2014). These duplications can allow for the emergence of new or divided functions between the new genes through mutation. This process is called neofunctionalization and sub-functionalization respectively and is believed to be the case for a variety of molecular structures such as trypsin/chymotrypsin (Baptista et al., 1998), myoglobin/hemoglobin (Storz, Opazo, & Hoffmann, 2013), and immunoglobulins (Glasauer & Neuhauss, 2014; Hsu et al., 2006; Ohno, 1970). The exact origins and functions of the duplicates of tlr4 in zebrafish, termed tlr4ba, tlr4al, and tlr4bb, are currently uncertain. A study by Sullivan et al. proposed that a tlr4 precursor was present in an ancient common ancestor, but both humans and zebrafish evolved their own current tlr4 genes through a series of gene loss and gain, making them paralogous to one another (2009). However, a recent study by Loes et al. suggests that the gain of tlr4 occurred prior to the divergence of ray-finned and lobe-finned fishes, meaning it would be orthologous to mammalian tlr4 (2021). A tandem duplication event then led to the emergence of tlr4ba and tlr4bb, and finally a species-specific duplication of tlr4ba in zebrafish led to the emergence of a third ohnolog, tlr4al. The unknown function of these homologs further confounds their origin, as both of studies have conflicting

results regarding their relation to LPS signalling (Loes et al., 2021; Sullivan et al., 2009). This is not surprising as duplications result in a lack of selective pressure for maintaining both genes, and due to the convoluted evolution of Tlr4 in fish, this likely resulted in functional divergence from their original ancestral gene product. Further inquiry into their function will help shed light on TLR4's puzzling origin and relation to human TLR4.

Zebrafish Tlr4 expression appears to vary depending on the homolog. On the other hand, humans express TLR4 in their peripheral blood lymphocytes, adipocytes, brain, endothelium, heart, intestines, kidneys, liver, lungs, ovaries, pancreas, placenta, prostate, spleen, testis, and thymus (Vaure & Liu, 2014). Zebrafish appear to express Tlr4ba in their blood, intestines, testis, skin, brain, liver, and heart, while Tlr4bb is expressed only within the blood, skin, and heart (Jault, Pichon, & Chluba, 2004). Little information can be found on where Tlr4al is expressed however, it was found to be upregulated in response to infection by Aeromonas salmonicida (Cornet et al., 2020). Interestingly, these genes are all found within the same cluster on chromosome 13 but are expressed in different areas of the body (See Chapter 2). Examining gene expression control could provide insight into their specific functions and why some tissues express certain homologs, while others do not. Fortunately, for the purpose of this project, both Tlr4ba and Tlr4bb appear to be expressed within the skin, and it was recently found that Tlr4ba is expressed within zebrafish hair cells using RNA sequencing (Barta et al., 2018). This supports using the lateral line as a model for CIO and examining therapies associated with TLR4.

1.4.4 *Toll-like receptor structure*

TLRs are class 1 transmembrane receptors, meaning they usually contain three main structural domains: the ectodomain, also known as the leucine-rich repeat (LRR) repeat motif, an alpha helix transmembrane domain, and an intracellular cytoplasmic TIR domain (Figure 1.4)

(Gay & Gangloff, 2007; Nie et al., 2018). This three-domain structure for TLRs first appears in the phylum Cnidaria, demonstrating its conservation throughout evolution (Carlos G.P. Voogdt, 2016; Liu et al., 2020). The ectodomain is located either outside the cell or within endosomes and acts to recognize and bind various ligands specific to the TLR. In mammals, TLR1, -2, -4, -5, -6 and -10 are localized to the plasma membrane, while TLR3, -7, -8, and -9 are found within intracellular vesicles (Sameer & Nissar, 2021). The ectodomain of TLRs contains approximately 16-29 LRRs that determine its structure and specificity (Botos, Segal, & Davies, 2011; Quiniou, Boudinot, & Bengtén, 2013). The LRR amino acid sequence has hydrophobic residues spaced in specific intervals, producing both α-helices and β-strands resulting in a concave hook shape that is conserved as far back as jawless vertebrates (Han et al., 2008; Kim et al., 2007; Matsushima et al., 2007; Quiniou, Boudinot, & Bengtén, 2013). The LRRs provide specificity to the TLR by the number, length, and sequence of LRRs as well as the amount of N-linked glycosylation within the ectodomain. The LRR repeats can be divided into two categories: a variable segment (VS) and a highly conserved segment (HCS) (Matsushima et al., 2007). TLR4 contains 22 LRRs and has approximately 5 N-glycosylation sites (Botos, Segal, & Davies, 2011; Ohto et al., 2012). In vertebrates, the ends of the LRRs are often capped by a LRR N-termini (LRRNT) and LRR C-termini (LRRCT), which protect the hydrophobic core of the ectodomain (Behzadi, Garcia-Perdomo, & Karpinski, 2021; Kang & Lee, 2011; Manavalan, Basith, & Choi, 2011; Matsushima et al., 2007). Dimerization of cell surface TLRs is dependent on the interaction between juxtamembrane sequences within their ectodomains of adjacent TLRs (Behzadi, Garcia-Perdomo, & Karpinski, 2021). The binding of a ligand allows for a conformational change that makes these motifs available for binding. Furthermore, endosomal TLRs require cleavage by

cathepsins (B, S, L, H, K) and asparaginyl endopeptidase before having the ability to dimerize after ligand binding (Behzadi, Garcia-Perdomo, & Karpinski, 2021; Fitzgerald & Kagan, 2020).

The structure and function of the transmembrane domain (TMD) of TLR molecules are relatively understudied compared to the ecto- and TIR domains. The TM domain of human TLRs is a hydrophobic α-helix and contains both a TM region and an intercellular linker region (ICL) (Mineev et al., 2017). The TMD interacts with the membrane interior through lipid-protein interactions and is likely implicated in the localization of the TLR within the cell, its movement within the membrane, and its activation (Kornilov et al., 2023; Mineev et al., 2017; Reuven, Fink, & Shai, 2014). There is a high level of amino acid conservation within the ICL of human TLRs, hinting at their crucial functional role within the cell.

The TIR domain is present on many proteins and is well conserved in animals, plants, and bacteria, demonstrating their long and conserved function in protein signalling (Burch-Smith & Dinesh-Kumar, 2007; Ve, Williams, & Kobe, 2015). The TLR TIR domain is composed of five parallel beta-sheets surrounded by five alpha helixes with three conserved “box” motifs (Behzadi, Garcia-Perdomo, & Karpinski, 2021; Ve, Williams, & Kobe, 2015). These box motifs are involved in structural stability and binding, and previous studies have established a greater than 50% sequence similarity in the TIR domains of TLRs (Ve, Williams, & Kobe, 2015). After TLR dimerization, the TIR domain recruits and binds various adaptor proteins, often through their own TIR domains. The adaptor proteins then interact with intracellular kinases to induce cellular responses and/or trigger the release of antimicrobial peptides, cytokines, chemokines, and ROS (Luo et al., 2020). The endoplasmic reticulum synthesizes TLRs and is responsible for assigning their specificity through glycosylation. Their folding and transportation within the cell

are provided by chaperones including, but not limited to GP96, PRAT4A, and Unc93B1 (Behzadi, Garcia-Perdomo, & Karpinski, 2021; Fitzgerald & Kagan, 2020).

Six TLR adaptor proteins interact through their TIR domains: myeloid differentiation primary response gene 88 (MyD88), Toll/interleukin-1 domain-containing adaptor protein (TIRAP/MAL), TIR domain-containing adaptor protein inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α and armadillo-motif containing protein (SARM), B-cell adaptor for phosphoinoditide 3-kinase (BCAP), and one TLR adaptor that interacts through TIR-non-TIR interactions: SLP65/76 and Csk-interacting membrane protein (SCIMP) (Behzadi, Garcia-Perdomo, & Karpinski, 2021; Luo et al., 2020; O'Neill & Bowie, 2007; Ve, Williams, & Kobe, 2015). These adaptors can be split into two categories: MyD88, TIRAP, TRIF, and TRAM constitute canonical adaptor proteins that are imperative in signal induction, while SARM, BCAP and SCIMP comprise the regulatory adaptors responsible for modulating TLR responses (Luo et al., 2020). Other TLR regulatory proteins indirectly modulate TLR responses, such as C5a anaphylatoxin chemotactic receptor 1 (C5aR1), which was reported to synergize with TLR4, increasing proinflammatory signalling in mice (Hernandez et al., 2017; Shi et al., 2021). On the other hand, low-density lipoprotein 1 (LRP1) is activated by TLR4 signalling to reduce inflammatory responses from macrophages (Luo et al., 2018). All TLRs use MyD88 except TLR3, however TLRs expressed on the plasma membrane most often signal through MyD88 and TIRAP adaptor proteins, while endosomal TLRs use TRIF and TRAM (Luo et al., 2020; Ve, Williams, & Kobe, 2015). However, some TLR proteins, such as TLR4, can be internalized upon ligand binding, utilizing plasma membrane and endosomal targeted adaptors (Luo et al., 2020).

1.4.5 The Toll-like receptor 4 signalling pathway

The discovery of TLRs stems from the Toll receptor first discovered in Drosophila, which was originally found to contribute to the fly's development through the ligand Spatzle, but was later studied for its involvement in sensing of fungal infections (Lemaitre et al., 1996; Medzhitov, Preston-Hurlburt, & Janeway, 1997). Today, TLRs are primarily known to respond to PAMPs, providing the basis for an innate immune response by release of antimicrobial peptides, proinflammatory cytokines and chemokines that promote proliferation, differentiation, and migration of other immune cells (Carlos G.P. Voogdt, 2016). On top of PAMPs, TLRs can also be activated by other types of molecular patterns, including endogenous DAMPs, microbial associated molecular patterns (MAMPs), and xenobiotic-associated molecular patterns (XAMPs) (Behzadi, Garcia-Perdomo, & Karpinski, 2021). These ligands often indicate cell damage from injury, non-pathogenic molecules, and threatening foreign bodies such as drugs and toxins.

TLR4 was the first identified member of the TLR family and is found both on the cell surface and within endolysosomes. TLR4 is unique for many reasons, but critically, it is the only known TLR to activate two separate signalling pathways responsible for vastly different activities (Rostamizadeh et al., 2022). It is the only TLR capable of secreting interferon 1 (IFN-1), and only one of two TLRs to use the TRAM adaptor protein. In mammals, TLR4's primary ligand is lipopolysaccharide (LPS), which allows for a prompt response to infection by gram-negative bacteria (Gauthier, Rotjan, & Kagan, 2022; Lu, Yeh, & Ohashi, 2008). During TLR4 signalling with LPS, the gram-negative cell wall is fragmented into LPS monomers by LPS-binding protein (LBP). This soluble protein facilitates the association of LPS to either soluble or a membrane-anchored form of cluster differentiation 14 (CD14). CD14 then transfers an LPS monomer to the TLR4/MD-2 heteroduplex, leading to homodimerization between a second TLR4/MD-2/LPS

complex through the lipid A motif within LPS. The dimerization of the TIR domains recruits the adaptor proteins: TIRAP or TRAM.

In humans, TLR4-LPS signalling occurs through two pathways: myeloid differentiation primary response 88 (MyD88)-dependent and MyD88-independent (TRIF) pathway (Figure 1.5). In the early phases of TLR4 activation, the MyD88-dependent pathway is activated by TIRAP interacting with TLR4 via its own TIR domain at the cell membrane (Gauthier, Rotjan, & Kagan, 2022; Kagan, Magupalli, & Wu, 2014; Kawai & Akira, 2007). This recruits a supramolecular organizing centre (SMOC) called the myddosome, which is composed of six MyD88, four interleukine-1 receptor-associated kinase 4 (IRAK4), and four IRAK2 death domains (DD) (Fitzgerald & Kagan, 2020; Kusiak & Brady, 2022). Myddosome formation contributes directly to the signal intensity and is dependent on the binding of TIRAP to TLR4 (Tang et al., 2023). Myddosome formation begins with an oligomer of MyD88 recruited through its C-terminal TIR domain to TIRAP (Kagan, Magupalli, & Wu, 2014; Lin, Lo, & Wu, 2010). MyD88 then binds to IRAK4 via its DD, followed by IRAK1 and IRAK2 (Kawasaki & Kawai, 2014; Kusiak & Brady, 2022). Activation of the myddosome through interaction with TIRAP and the DDs of IRAK4 leads to phosphorylation of IRAK1 and IRAK2 by IRAK4. Tumour necrosis factor receptor-associated factor 6 (TRAF6) then binds to this complex, allowing the IRAK kinases along with TRAF6 to dissociate from the myddosome (Kusiak & Brady, 2022). TRAF6, an E3 ubiquitin ligase polyubiquitinates itself and recruits a complex consisting of transforming growth factor-β-associated kinase-1 (TAK1) and TAK1 binding proteins 1, 2, and 3 (TAB1-3). TRAF6, along with other ubiquitin-conjugating enzymes, polyubiquitinates TAB2 and TAB3, which activates TAK1(Kawasaki & Kawai, 2014). TAK1 phosphorylates the IKK complex (comprised of IKKα, IKKβ, and IKKγ) as well as the mitogen-activated protein kinase (MAPK) pathway (Fitzgerald

& Kagan, 2020; Kawai & Akira, 2007; Kawasaki & Kawai, 2014; Kusiak & Brady, 2022). The IKK complex phosphorylates IκB proteins attached to NFκB, leading to their degradation by the proteasome (Kawai & Akira, 2007; Kusiak & Brady, 2022; Zhang et al., 2021). This unmasks the nuclear localization signal on NFκB, allowing for its translocation to the nucleus and transcription of proinflammatory genes. Simultaneously, the TAK1-activated MAPK pathway phosphorylates activating protein-1 (AP-1), allowing for translocation to the nucleus and transcription of inflammatory genes (Kawasaki & Kawai, 2014). Proinflammatory mediators released from NFκB and AP-1 include tumour necrosis factor α (TNFα), interleukin (IL)-6, cyclooxygenase 2, and type III interferons (IFNλ1/2). IL-10 is also released during NFκB activation, an anti-inflammatory cytokine that limits the overproduction of inflammatory cytokines.

Approximately 15-45 minutes after TLR4 activation by LPS at the plasma membrane, endocytosis of the LPS/TLR4/MD-2/CD14 complex occurs in a CD14 and MD-2-dependent manner, terminating the MyD88-dependent signalling (Figure 1.5)(Ciesielska, Matyjek, & Kwiatkowska, 2021; Kagan et al., 2008; Tanimura et al., 2008; Tsukamoto et al., 2018; Zanoni et al., 2011). TRIF-related adaptor molecule (TRAM) contains an N-terminal myristoylation site that localizes it to the Golgi membrane and plasma membrane, where it surveys for dimerized TLR4 (Rowe et al., 2006). Once the TLR4 complex is endocytosed, TRAM translocates with the endosome, interacting with the TIR domain of TLR4 via a TIR:TIR domain interaction. TRAM mainly acts as a bridging protein for the formation of the triffosome, a SMOC signalling complex like the myddosome (Verstak et al., 2014). Although the triffisome remains poorly defined, after TRIF is recruited to TRAM, it interacts with TRAF3. TRAF3 recruits and phosphorylates TANK-binding kinase 1 (TBK1) and inducible IκB kinase ε (IKKi), which

phosphorylates interferon regulatory factor 3 (IRF3) (Brown et al., 2011; Fitzgerald & Kagan, 2020). IRF3 then translocates to the nucleus, leading to the expression of type 1 interferons such as IFN-β (Brown et al., 2011; Fitzgerald & Kagan, 2020; Sheedy & O'Neill, 2007). TRAM also binds to TRAF6 via an N-terminal TRAF-6 binding motif, which may contribute to the optimal expression of proinflammatory cytokines through both the MyD88-dependent and Myd88-independent pathways (Kawai & Akira, 2010; Verstak et al., 2014). Finally, TLR4 is degraded as the endosome transitions into a lysosome, terminating signalling (Ciesielska, Matyjek, & Kwiatkowska, 2021).

1.4.6 Ligands of TLR4

1.4.6.1 Bacterial ligands

Coordinate with the position of TLR4 on the plasma membrane for environmental sensing, most TLR4 ligands are found on the outermost layer of an organism, such as the outer membrane, structural proteins, cell wall, or excreted products (Gay & Gangloff, 2007). As mentioned previously, TLR4 signals through various ligands ranging from endogenous DAMPs to xenobiotic molecules. For example, vertebrate TLR4 recognizes bacterial ligands beyond LPS, including teichuronic acid and mannuronic acid polymers, components of gram-positive and gram-negative bacterial cell walls, respectively (Flo et al., 2002; Yang et al., 2001). Heat shock protein 60 (HSP60), is widely expressed in prokaryotes and eukaryotes and regulates a multitude of cellular events (Y. Duan et al., 2020). HSP60 is found on the outer membrane of Chlamydia spp. during persistent infection and mediates TLR4 activation, resulting in apoptosis within the reproductive tract (Da Costa et al., 2004; Equils et al., 2006). Multiple ligands other than LPS from *Pseudomonas aeruginosa* have also been reported to activate TLR2 and TLR4 (McIsaac, Stadnyk, & Lin, 2012). Exoenzyme S (ExoS) and the capsule of P. aeruginosa likely activate

TLR4 through the MyD88-dependent pathway, while Slime-GP activates TLR4 through the MyD88-independent MAPK pathway (Epelman et al., 2004; Flo et al., 2002; Lagoumintzis et al., 2008). The ligands mentioned above act as TLR4 agonists, but other bacterial ligands of TLR4 act as antagonists by competing with or inhibiting LPS binding to co-receptor molecules of TLR4 (Molteni, Bosi, & Rossetti, 2018). Lipid A within LPS from *Rhodobacter sphaeroides*, lipopoligosaccharides (LOS) from *Bartonella quintana*, and CyP lipid A from *Oscillatoria planktothrix* FP1 all act as inhibitors of TLR4 signalling (Kutuzova et al., 2001; Macagno et al., 2006; Malgorzata-Miller et al., 2016).

1.4.6.2 Viral ligands

Activation of TLR4 from viruses often leads to immune overactivation and cytokine storms, where the body can not handle the large number of cytokines produced, furthering disease severity (*National Cancer Institute*, 2023; Olejnik, Hume, & Muhlberger, 2018). Different viruses often activate TLR4 through their membrane or capsid-associated proteins. The membrane F protein from the respiratory syncytial virus (RSV) stimulates TLR4 in a CD14 and MD-2-dependent manner, resulting in activation of NFκB and proinflammatory cytokine release (Kurt-Jones et al., 2000; Rallabhandi et al., 2012). The core structural protein lipopeptides in human hepatitis C virus (HCV) activates both TLR2 and TLR4, upregulating NFκB activity (Duesberg et al., 2002), and the Ebola virus glycoprotein on the capsid binds to the TLR4/MD2 complex increasing release of proinflammatory cytokines (Lai et al., 2017; Okumura et al., 2010). The glycoprotein G on vesicular stomatitis virus (VSV) also mediates TLR4 activation through a MyD88-independent pathway involving TRAM and IRF7, leading to the expression of type 1 interferons (Georgel et al., 2007).

Unlike the structural proteins and related glycoproteins on the viruses above, dengue virus infected cells release the virulence factor non-structural protein 1 (NS1), which activates TLR4 on endothelial cells, leading to a massive proinflammatory response and ROS generation (Dominguez-Aleman et al., 2021). Another protein, Tat, produced by the human immunodeficiency virus 1 (HIV-1) activates TLR4 in mononuclear phagocytes (Ben Haij et al., 2015). The phagocytes produce large amounts of proinflammatory cytokines through NF-κB induction and Ben Haij et al. proposes multiple mechanisms in this inflammatory environment facilitates disease progression to AIDS (2015). Furthermore, human cytomegalovirus (HCMV) mediates TLR4/MD2/CD14 activation through the MyD88-dependent and MyD88-independent pathways, however, the exact component responsible for this activation is unknown (Marques, Ferreira, & Ribeiro, 2018; Yew et al., 2012).

Recently, the involvement of TLR4 in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection has been examined, showing that TLR4 binds to the S1 spike protein leading to proinflammatory cytokine release and later, an upregulation in angiotensin-converting enzyme 2 (ACE2) production (Aboudounya & Heads, 2021). ACE2 is a receptor on host cells used by SARS-CoV-2 to initiate viral entry, therefore TLR4 may be involved in increased viral uptake. Other observations of viruses exploiting TLR4, such as the mouse mammary tumour virus, uses TLR4 to replicate in B-cells, showing that viral interactions with TLRs are not always directed towards regular innate immune surveillance and proinflammatory responses (Jude et al., 2003; Pichlmair & Reis e Sousa, 2007). However, due to TLR4's ability to prime the adaptive immune response to infections, viral ligands are being examined as therapeutic vaccine adjuvants (Sartorius et al., 2021).

TLR4 is also activated by multiple different eukaryotes, apart from viruses and prokaryotic organisms like bacteria and cyanobacteria, including fungi, protozoa, nematodes, cestodes and even plants. O-linked mannosyl, a component of the outer cell wall in the fungal pathogen *Candida albicans*, binds to TLR4 and engages both the MyD88-dependent and MyD88-independent pathway leading to the release of TNF, IL-6, IL-1, and CXCL-1/2 (Bojang et al., 2021; Wang et al., 2019). Wang et al. also shows that Sel1, a small, secreted cysteine-rich protein (SCP) released during *Candida* infection, acts as a ligand for both TLR4 and TLR2. Other fungal PAMPs, such as the conidia from *Aspergillus fumigatus* and glucuronoxylomannans (GXMs) from the capsule of *Cryptococcus neoformans* are also capable of inducing TLR4 activation or inhibiting the proinflammatory response of TLR4 (Behzadi, Behzadi, & Ranjbar, 2016; Chai et al., 2011; Hatinguais, Willment, & Brown, 2020).

1.4.6.3 Protozoan ligands

The surface glycoconjugates known as glycosylphosphatidylinositol (GPI) anchors, and it's related molecules (lipophosphoglycan (LPG) and glycosylinositol phospholipids (GIPLs)) on parasitic protozoans, such as Plasmodium falciparum, Toxoplasma gondii, Trypanosoma cruzi, Leishmania major, and Entamoeba histolytica all share a similar structure and play an essential role in their interaction with the host (Ropert & Gazzinelli, 2000). GIPLs are free GPI anchors not linked to a protein or carbohydrate, while LPGs are GPI anchors linked to heavily phosphorylated glycan repeats (Ropert & Gazzinelli, 2000). The glycocalyx of T. cruzi heavily populated by GPI-anchored mucin-like glycoproteins (GPI-mucins) and GIPLs, with the latter acting as a ligand of TLR4 inducing NF-κB activation, resulting in proinflammatory cytokine release and an increase in ROS and NO (Cerbán et al., 2020; Oliveira et al., 2004; Rodrigues, Oliveira, & Bellio, 2012). Whether GIPLs from T. cruzi induce activation of NF-κB through the

MyD88-dependent or independent pathway is unclear, however, an interesting finding by Rodrigues et al. suggests eGIPL may interact with TLR4 independently of CD14 (2012). Furthermore, GPI in P. falciparum, T. gondii, and E. histolytica act as ligands for both TLR2 and TLR4, either promoting a protective response or potentially facilitating pathogenesis in the case of P. falciparum (Debierre-Grockiego et al., 2007; Denkers, 2010; Krishnegowda et al., 2005; Maldonado-Bernal et al., 2005; Zare-Bidaki et al., 2014).

Additionally, the surface protein trypomastigote surface antigen (TSA-1) expressed in T. cruzi, was also found to mediate TLR4 activation through the MyD88-dependent pathway (Amith et al., 2010). It's crucial to note that this was not due to TSA-1 acting as a ligand, but rather the removal of a sialyl residue, allowing for dimerization and activation. Another protein, HSP70 in T. gondii, activates TLR4 through both the MyD88-dependent and MyD88-independent pathway and is reported to be involved in a diverse set of actions such as maturation of DCs, anaphylactic onset, and neuroinflammation (Cheng et al., 2020).

1.4.6.4 Helminth ligands

Parasitic helminths elicit a type 2 cytokine response that begins with epithelial cells, which involves many adaptive and innate immune cells (Oyesola et al., 2020). This response recruits multiple mechanisms to promote the removal of the parasite and healing of tissues damaged during the infection, unlike in a typical type 1 cytokine response that promotes inflammation in attempts to kill intracellular parasites (Berger, 2000; Oyesola et al., 2020). A study by Tawill et al. demonstrated that glycans on excreted proteins are one of the antigens driving the type 2 cytokine response in parasitic schistosomes and nonparasitic nematodes (2004). Particularly, the glycoprotein lacto-N-fucopentaose III (LNFPIII) found on the eggs of many helminth parasites, was found to activate DC2s (an APC for T helper 2), through the

binding of TLR4, leading to the release of type 2 cytokines (McDonald, 2009; Thomas et al., 2003; Tundup et al., 2015). Later research found that excretory-secretory (ES) molecules comprise a large body of immune-modulating products from helminths with varying effects on TLR4. The fatty-acid-binding protein (Fh15) from Fasciola hepatica acts as an antagonist of TLR4, preventing macrophages from developing a bias for type 1 cytokines (Lothstein & Gause, 2021).

When there are defence mechanisms evolved against pathogens, there will always be parasites finding ways to exploit these systems. Acanthocheilonema Viteae uses ES-62 (a secreted phosphorylcholine glycoprotein) to bind TLR4 and down-regulate the levels of proinflammatory cytokines by inhibiting the maturation of APCs (Pineda et al., 2014). The binding of ES-62 to TLR4 on mast cells also reduces degranulation and therefore, inflammation. Other ES proteins such as Ts soluble products (TsSPs) from Trichuris suis and tegmental coat antigens of F. hepatica indirectly reduce expression of TLR4 or supress TLR4 activation but can not be considered ligands of this receptor (Lothstein & Gause, 2021; Maizels, Smits, & McSorley, 2018).

1.6.4.5 Plant ligands

The number of compounds in plant extracts associated with antagonism and modulation of TLR4 to induce anti-inflammatory effects is extensive (Coutinho-Wolino et al., 2022; Kuzmich et al., 2017; Oledzka & Czerwinska, 2023). Many of these compounds down-regulate TLR4 expression or inhibit TLR4 signalling by binding to co-receptors such as MD-2 or a downstream signalling molecule such as TRAF6 or IKK proteins. For example, curcumin, a yellow pigment found in turmeric, and berberine, an alkaloid found in Rhizoma coptidis, can bind to MD-2, preventing the formation of the CD14/MD-2/TLR4 complex, supressing the

release of proinflammatory cytokines in the presence of LPS (Chu et al., 2014; Gradisar et al., 2007). Iberin, an analogue of the known TLR4 inhibitor sulforaphane, was isolated from cabbage and other vegetables and prevented the dimerization of TLR4 by binding cysteine residues, preventing its activation (Shibata et al., 2014). On the other hand, some plant proteins act as defence molecules to prevent their consumption and can promote inflammation. For example, plant lectins such as Pinellia ternata lectin (PTL), ricin toxin, and mistletoe have been shown to bind TLR4, leading to the activation of innate immune cells and downstream inflammatory pathways (Dong et al., 2020; J. Li et al., 2023; Park et al., 2010). The Yew extract paclitaxel (taxol) is the most well-known naturally occurring anti-cancer drug and was later found to promote its effects, in part, by acting as a TLR4 agonist. Activation of TLR4 by taxol increases the release of anti-tumour cytokines and promotes DC and M1 macrophage maturation and activation, reducing tumour progression (Yu et al., 2022).

1.4.6.6 Endogenous ligands

Some of the most important TLR4 ligands are endogenous and signal tissue or cell injury to the immune system, recruiting immune cells for repair and fighting the infection. Endogenous TLR ligands, also called alarmins, can be released in one of two ways: 1) the cell is injured and passively releases cellular components that warn the immune system of injury or ongoing inflammation, or 2) the cell actively secretes molecules to purposefully attract innate and adaptive immune cells to a site of infection. TLR4 plays the most prominent inflammatory and innate immune cell signalling role for DAMPs, and their significance in autoimmune disease has been extensively reviewed within the literature (Yu, Wang, & Chen, 2010). HMGB1 is a DAMP of critical importance, as it can act as a potent stimulator of TLR4 and plays a critical role in cancer development and therapy (Wang & Zhang, 2020). Concerning TLR4 activation, HMGB1

must first be released by the cell either through active secretion, apoptosis, or necrosis (Wang & Zhang, 2020). HMGB1 begins within the nucleus in a fully reduced form with three key cysteine residues (Cys23, Cys45, Cys106) expressing thiol groups (Yang, Wang, & Andersson, 2020). When two of these cysteine residues (Cys23, Cys45) become oxidated, they bind to one another, turning HMGB1 into a potent ligand of TLR4 on macrophages, leading to the release of cytokines such as TNF-α, IL-1, and type 1 IFNs (Yang et al., 2015). Only in this partially oxidized state can HMGB1 activate TLR4 in combination with MD-2 (Xue et al., 2021).

1.4.6.7 Metal ligands

As will be discussed in more detail in Chapter 2, metals such as nickel (Ni), cobalt (Co), palladium (Pd) and platinum (Pt) are somewhat newly discovered ligands of TLR4 (Domingo et al., 2023; Rachmawati et al., 2013; Schmidt et al., 2010). Nickel and cobalt have been observed to bind directly to the ectodomain of TLR4 through key histidine residues (H431, H456, H458), allowing for its homodimerization, recruitment of MD-2, and its subsequent activation (Oblak, Pohar, & Jerala, 2015; Peana et al., 2017; Schmidt & Goebeler, 2015). Although palladium and platinum have been observed to induce similar responses, it is currently unknown whether this is mediated through these same histidines (Schmidt & Goebeler, 2015). Regardless, the binding mechanism for metals is unique from the TLR4's primary ligand LPS, as MD-2 is not required, and dimerization occurs in its absence (Raghavan et al., 2012). This has been a critical observation in the understanding of metal-induced contact hypersensitivities, providing a mechanism through which these allergic reactions occur (Schmidt & Goebeler, 2018). Domingo et al. have recently demonstrated the platinum-based chemotherapeutic cisplatin, along with other platinum compounds, are also capable of direct binding of human TLR4, which was previously demonstrated by Babolmorad et al. to mediate its activation (2021; 2023). This

suggests a novel pathway through which cisplatin induces inflammation and increases ROS generation, as well as a novel mechanism for the generation of therapeutics to mitigate inflammation generated by cisplatin toxicity.

1.4.6.8 Summary

The diversity of ligand type and signalling pathways utilized by TLR4 in response to bacteria, viruses, fungus, drugs, and endogenous ligands demonstrates TLR4's versatility and importance in pathogenic ligand recognition and initiation of the innate immune response. Although TLR4 is known to be activated to help signal infection to the body, many of these organisms have found ways to exploit TLR4 to further infection, exhibiting some co-evolutionary relationship between the innate immune system and pathogens. Therefore, determining the conservation in the binding of these ligands between species may provide insight into the evolutionary relationships of TLR4 function. For example, bacterial ligands such as LPS function as a ligand for TLR4 in mammals, but likely do not in other species such as fish. DAMPs, on the other hand, appear to function as TLR4 ligands in mammals (ex. HMGB1) as well as within invertebrates such as fruit flies (ex. Spatzle) (Nie et al., 2018). Furthermore, many of these ligands involve using different co-receptors to induce a response. When considering evolutionary relationships between TLR4 of different species, it would be prudent to examine which co-stimulatory molecules are present and utilized within each species. To determine the possibility of metals acting on zebrafish Tlr4, we examine both structural similarities to human TLR4 and conservation in co-receptor molecules.

1.4.7 *Inhibition of Toll-like receptor 4*

There are three major ways to inhibit the overactivation of TLR4 signalling in the treatment of disease, including modulating TLR4 expression, inhibiting TLR4 signalling through

direct binding to TLR4 or the TLR4/MD-2 complex or inhibiting downstream adaptors and signalling molecules. Many TLR4 inhibitors have been developed for these purposes and have been previously reviewed in detail (Chen, Kao, & Liu, 2018; Kuzmich et al., 2017; Peri & Piazza, 2012; Romerio & Peri, 2020; Zaffaroni & Peri, 2018; Zhang et al., 2022). According Clinicaltrials.gov, there are currently 7 TLR4 inhibitors in clinical trials, including Eritoran, NI-0101, ApTOLL, JKB-122, EB05, E-5531 and TAK-242 (N. L. o. M. (N. L. o. M. (NIH), 2023). Eritoran and TAK-242 are among the most well-studied TLR4 inhibitors (Kuzmich et al., 2017). Eritoran is a lipid A analog that binds to MD-2, but due to its increased size in comparison to lipid A, it prevents the formation of the TLR4/MD-2 complex. The hydrophobic binding pocket of MD-2 is filled up 90% by Eritoran, and no direct interaction with TLR4 occurs (Kim et al., 2007). TAK-242 is a sulfonamide linker that binds intracellularly to the TIR domain of TLR4 through Cys747. This prevents the association of adaptor molecules TIRAP and TRAM, preventing downstream signalling and transcription of proinflammatory genes (Kuzmich et al., 2017). Due to subpar results of TAK-242 in clinical trials (Rice et al., 2010), there has been recent development of TAK-242 derivatives and nitrobenzyl prodrugs that show high efficacy inhibiting TNF-α and ROS (Lee et al., 2020; Plunk et al., 2020).

Due to the involvement of inflammation, proinflammatory cytokines, chemokines, and ROS in many diseases and their progression, there is a high therapeutic potential for inhibiting TLR4. The disease most associated with inhibiting TLR4 is sepsis, which LPS generates from prolonged gram-negative bacterial infections (Heine & Zamyatina, 2023). Both Eritoran and TAK-242 were initially examined for their treatment of sepsis and showed promise, but unfortunately, both showed no improvement in 28-day mortality and failed in phase 3 clinical trials (Opal et al., 2013; Rice et al., 2010). However, inhibitors are still being examined in

treating other diseases, such as influenza and rheumatoid arthritis (Samarpita et al., 2020; Shirey et al., 2013). TLR4 also plays a dual role in cancer progression and treatment. Chronic inflammation through continued TLR4 activation and ROS generation leads to carcinogenesis, and its inhibition has shown promise in decreasing tumour development and progression (Chen, Kao, & Liu, 2018; Echizen et al., 2018). On the other hand, due to the high concentration of anti-inflammatory, immune cells in the tumour microenvironment (such as tumour-associated macrophages), activation of TLR4 has shown promise in suppressing tumour progression (Huang, Xu, & Peng, 2018; Smith et al., 2018). Therefore, when generating therapeutics affecting TLR4 in the treatment of cancer, it is imperative that regular proinflammatory function is maintained.

1.5 Objectives and purpose of thesis

The overall objective of this thesis was to examine Tlr4 function within zebrafish and its involvement in ototoxicity after treatment with the chemotherapeutic cisplatin. In Chapter 2, I examine the possibility of group 9/10 metals to act as novel ligands of the zebrafish Tlr4 homologs Tlr4ba, Tlr4al, and Tlr4bb. I hypothesize that group IX/X transition metals will facilitate zfTlr4 activation, leading to NFκβ induction and a proinflammatory response. In Chapter 3, I examine the efficacy of 14 novel derivatives of TAK-242 in reducing cisplatin-mediated neuromast hair cell death through zebrafish Tlr4. I hypothesize that cisplatin can activate zebrafish Tlr4 and be selectively inhibited, allowing for regular immune surveillance and canonical signalling by LPS to remain. Finally, in Chapter 4, I provide a general summary of thesis findings, future directions, and further discuss these findings in relation to current literature.

The purpose of this thesis is to improve our understanding of the innate immune receptor TLR4, both in its role within CIO and its functions within zebrafish, while providing insight into its evolutionary beginnings. Zebrafish possess many innate advantages over other animal models and are crucial in studying diseases, their related phenotypes, and treatments. To improve their application to human diseases, critically examining the similarities and differences with their mammalian counterparts is prudent for determining the relevance of any scientific finding. Moreover, due to the early divergence of bony fish from other vertebrates in the evolutionary timeline, conserved protein function and structure as well as their fundamental differences, can give insight into evolutionarily important processes for survival and fitness.

TLR4 is one example of a highly conserved innate immune receptor within a wide range of species, however, their function remains a mystery within zebrafish. The first aim was to determine the possibility of group 9/10 metals to act as ligands of zebrafish Tlr4 homologs: Tlr4ba, Tlr4al, and Tlr4bb. This was performed by examining their contribution to cell toxicity using fluorescent microscopy, as well as their activation when expressed in heterologous cell culture. Following the pioneering observations by Schmidt et al. (2010) that TLR4 acts as a direct receptor for nickel, as well as the observation by Babolmorad et al. (2021) that zebrafish Tlr4ba and bb knockdown reduced cell toxicity in the presence of cisplatin, we predicted zebrafish Tlr4 homologs were involved in group 9/10 metal ion sensing, leading to their activation.

The second aim was to determine if we could use novel inhibitory compounds to target Tlr4 and diminish CIO using zebrafish as a model. Due to their homologous nature to mammalian inner ear hair cells, zebrafish neuromast hair cells provide a unique structure for examining CIO in vivo. I used morphological and behavioural analyses to determine the

protective effects of TAK-242 derivatives during cisplatin treatment. We predict that inhibition of TLR4 will reduce inflammation and ROS generation within hair cells and their environment, therefore mitigating cisplatin toxicity.

1.6 Figures and Tables

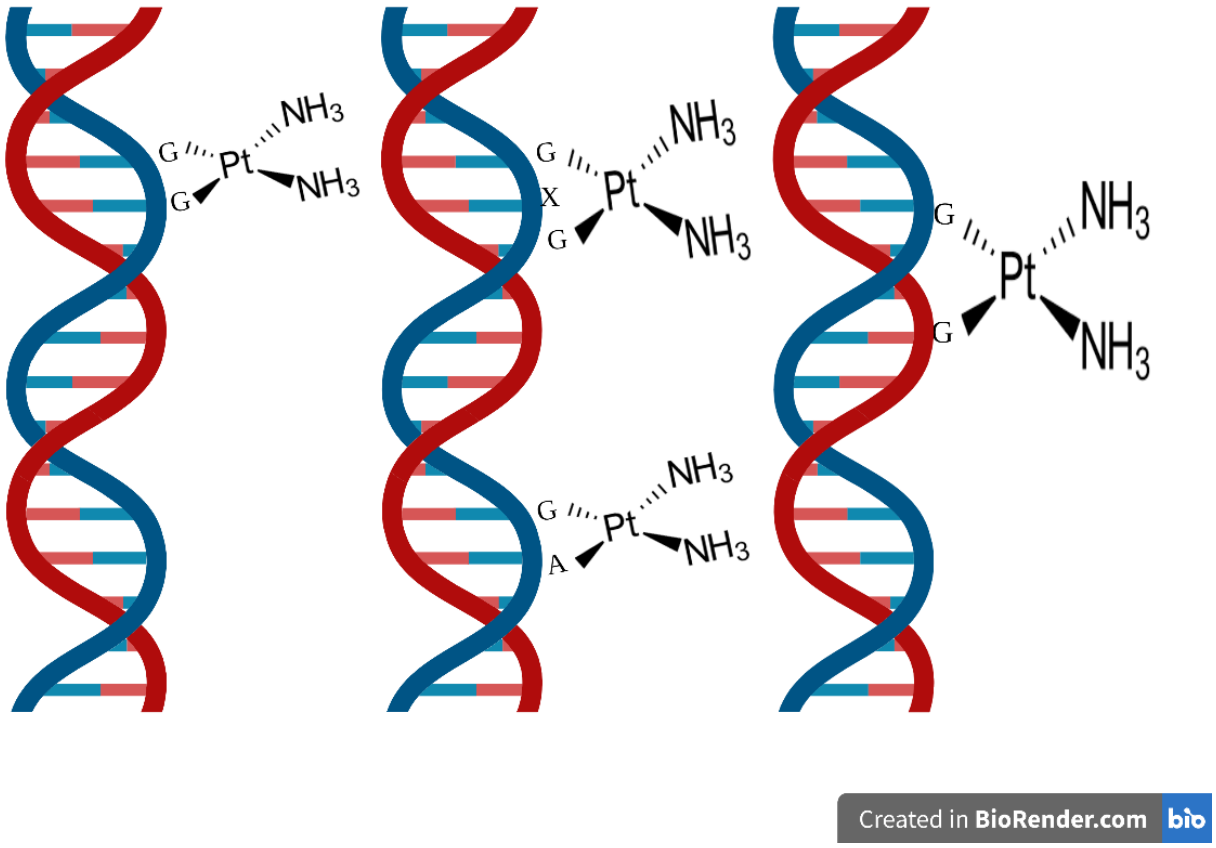


Figure 1. 1 Cisplatin binds to DNA leading to intra- and inter-strand crosslinks. Cisplatin preferentially binds to guanine and adenine forming 1,2-d(GpG), 1,2-d(GpA), and 1,3-d(GpXpG) cross links as well as inter-strand G-G crosslinks. Other inter- and intra-strand crosslinks are possible, but not shown here. Created with BioRender.

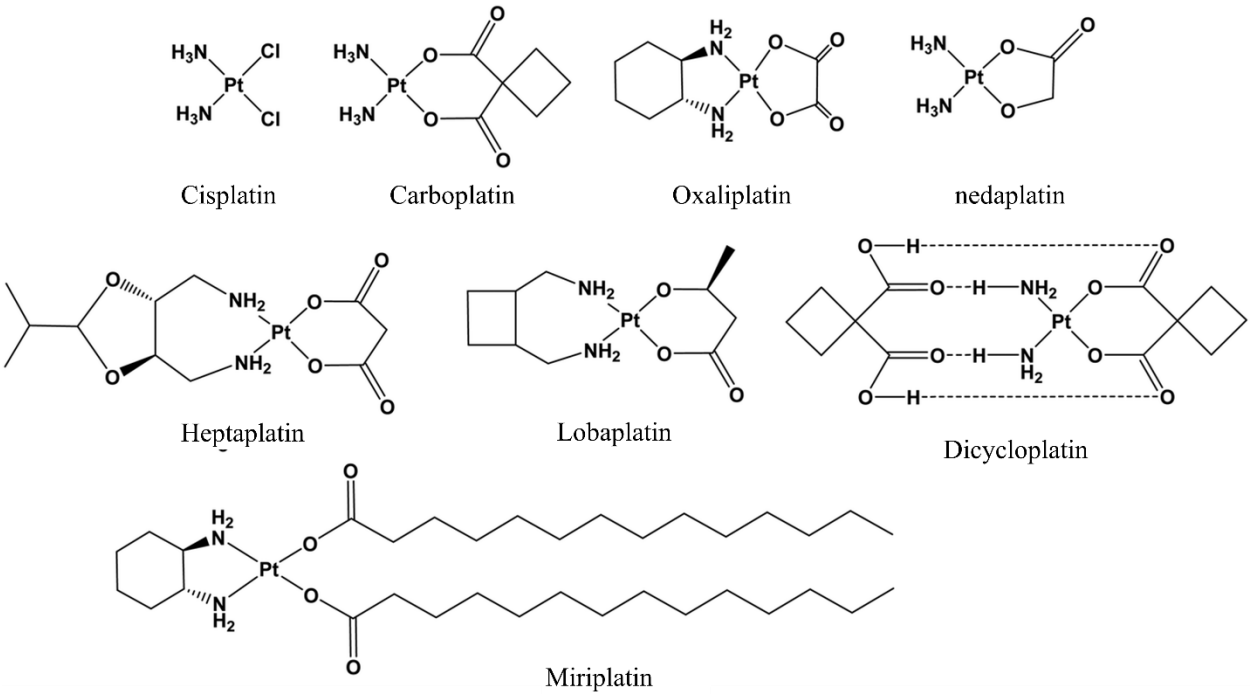


Figure 1. 2 Chemical structures of platinum based anticancer agents in clinical use.

Cisplatin, Carboplatin, and Oxaliplatin are clinically approved worldwide, while the other four are approved in select Asian countries.

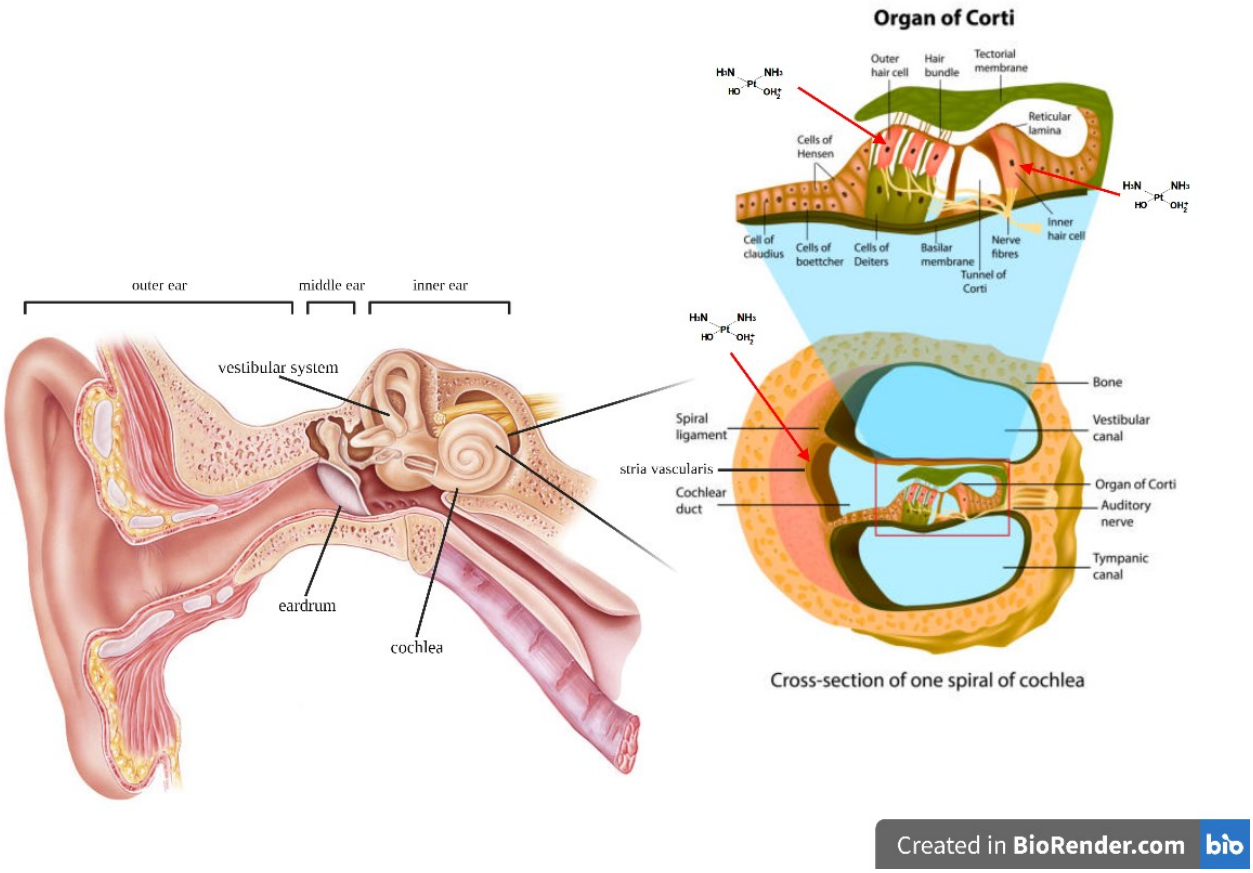


Figure 1. 3 The human ear and structures affected by cisplatin. Schematic of the human auditory system depicting structures affected by cisplatin. Cisplatin enters the inner ear through an unknown mechanism, but preferentially damages the stria vascularis and hair cells within the organ of Corti. Red arrows point to structures affected by cisplatin. Figures are not to scale. Created with BioRender.

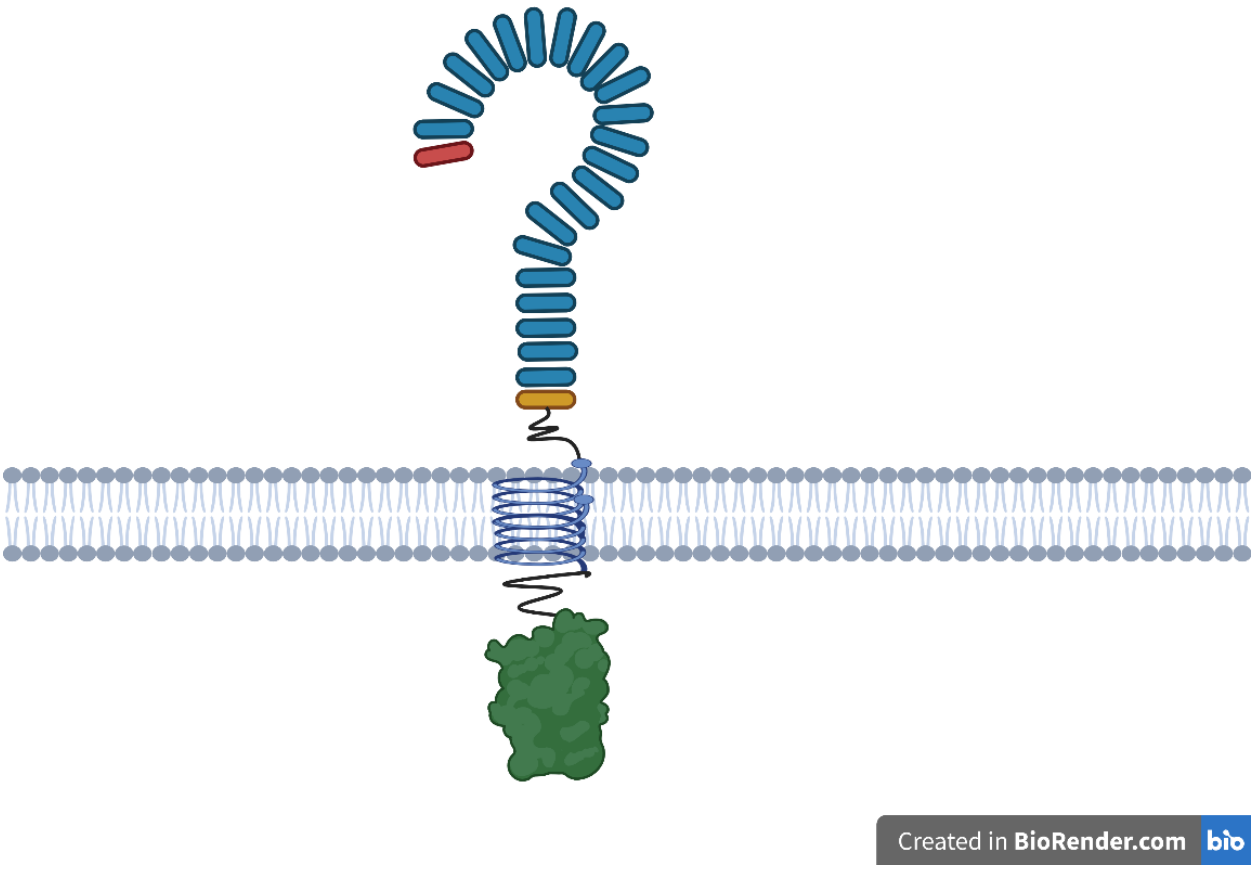


Figure 1. 4 Diagram of the structure of TLR4. Blue oval structures represent LRRs. Red and orange oval structures represent the LRRNT and LRRCT respectively. Blue spiral represents the intermembrane domains, while the green protein attached represents the intracellular TIR domain. Created with BioRender.

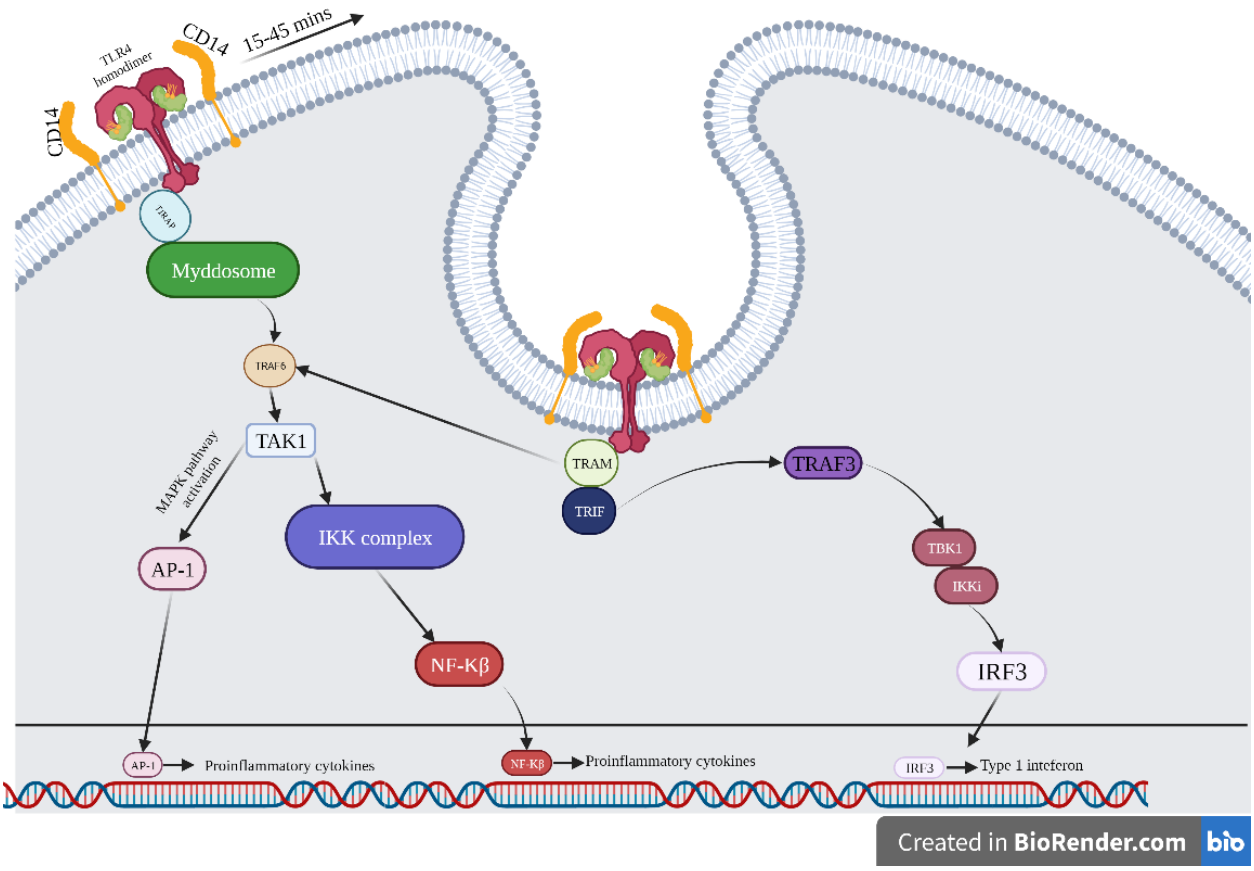


Figure 1. 5 TLR4 pathway following LPS activation. TLR4 binds to MD-2 (light green) and an LPS monomer. This leads to its dimerization and initiates signalling through the MyD88-dependent pathway. This culminates in the transcription of proinflammatory genes by NF-Kβ and AP-1. Approximately 15-45 minutes later, the TLR4 dimer is endocytosed in a CD14 dependent manner. This terminates signalling through the MyD88-dependent pathway and initiates signalling through the MyD88-independent pathway. This pathway contributes to both proinflammatory cytokine production as well as type 1 interferon production by signalling through both TRAF6 and IRF3 respectively. Created with BioRender.

Table 1. 1 Summary of cisplatin side-effects and current therapies.

Side effect	Likelihood of development	Symptoms/diagnostic markers	Current and developing therapies	Reference
Gastrointestinal toxicity	○ 70-80% of patients	○ Nausea ○ Vomiting ○ Diarrhea ○ Metallic taste ○ Pancreatitis ○ Mucositis ○ Anorexia ○ Pica ○ Emesis ○ Weight loss ○ Delayed gastric motility ○ Mucositis ○ Malabsorption ○ Barrier impairment	○ 5-hydroxytryptamine (serotonin) receptor antagonists (ondasetron) ○ Cerenia ○ Dexamethasone ○ Lorazepam ○ Metoclopramide ○ Aprepitant ○ Fosprepitant ○ Caffeic acid ○ Chrysin ○ Glucagon-like peptide-2 (GLP-2) ○ Ghrelin ○ L-carnitine ○ AOB ○ Glutamine ○ Riboflavin ○ Folate ○ EDTA ○ Olanzapine ○ Ginger ○ Ferulic acid ○ Korean ginseng root extract ○ Low molecular fucoidan (LMF) ○ Flaxseed oil ○ Dunnione ○ 18β-Glycyrrhetinic acid (GA) ○ Nigella sativa oil ○ Thymoquinone ○ NSO and TQ	○ (Ghosh, 2019) ○ (Qi et al., 2019) ○ (Shahid, Farooqui, & Khan, 2018)
Hepatotoxicity	○ ~36% of patients	○ Elevated serum transaminases ○ Elevated serum alkaline phosphatase ○ Elevated lactate dehydrogenase ○ Elevated bilirubin ○ Elevated *c*-glutamyl transpeptidases ○ Abdominal pain ○ Abdominal swelling	○ Infliximab ○ Ethyl acetate extract ○ Green tea ○ Methanolic extract ○ Pine bark extract ○ Sorghum leaf sheath ○ Increased fruit intake ○ Curcumin ○ Apocynin ○ Proanthocyanidin ○ Daidzen ○ Baicalein ○ Hesperidin ○ Rutin ○ Kolaviron-defatted acetone extract ○ Ginger ○ Caffeic acid ○ Protocatechuic acid ○ Ellagic acid ○ Crocin ○ Ginseng ○ Garlic oil ○ Honey ○ Protocatechuic acid	○ (İşeri et al., 2007) ○ (Qi et al., 2019) ○ (Abd Rashid et al., 2021) ○ (Habib et al., 2021)
Cardiotoxicity	○ ~6% of patients	○ Electrocardiographic changes ○ Arrythmias ○ Myocardidtis ○ Cardiomyopathy ○ Congestive heart failure ○ High serum lactate dehydrogenase ○ High serum creatine ○ Pericarditis ○ Blood pressure changes ○ Heart attack ○ Cardiac failure ○ Angina	○ High doses of selenium and vitamine E ○ Reserveratrol ○ Apocynin ○ Alpha-lipoic acid ○ Propionyl-L-carnitine	○ (Ghosh, 2019) ○ (Al-Majed et al., 2006) ○ (Aldossary, 2019) ○ (Qi et al., 2019) ○ (Abd Rashid et al., 2021)
Myelosuppression	○ 25-30% of patients	○ Fever and infection ○ Thrombosis ○ Leukopenia ○ Neutropenia ○ Thrombocytopenia	○ Bone marrow transplant ○ Growth factor injections ○ Blood transfusions ○ Dose modification ○ Butorphanol ○ Cerenia ○ Erythropoietin ○ Quercetin	○ (Food and Drug Administration, 2019) ○ (Qi et al., 2019) ○ (Barabas et al., 2008) ○ (Chuang et al., 2022) ○ (Sinha et al., 2015) ○ (Henry et al., 1995) ○ (Masuda et al., 2009)

			○ Vetiver oil ○ Maitake β-glucan ○ Methanol extract (*C. deodara*)	○ (Sushmitha et al., 2022)
Nephrotoxicity	○ 28-36% of adult patients ○ 70% of pediatric patients	○ Higher concentrations of cisplatin within kidneys than the blood ○ Renal failure ○ High serum creatinine ○ High serum urea ○ Low glomerular filtration ○ Hypomagnesemia ○ Hypokalemia ○ Fibrotic nephropathy ○ Hypoalbuminemia	○ Pre-, during, and post-treatment hydration using hypertonic saline (3-6L per day) ○ Mannitol ○ Magnesium ○ Sodium thiosulfate (STS) ○ Amifostine ○ Dialysis ○ Cisplatin dose reduction ○ Plasmapheresis ○ Selenium ○ Vitamine E ○ Dimethylthiourea (DMTU) ○ Ebselen ○ Allopurinol ○ Tetrahydrocurcumin ○ Copper ○ Cimetidine ○ Probenecid ○ Acivicin ○ Diethyldithiocabamate ○ N-acetyl cysteine ○ IL-10 ○ siRNA against p53 ○ MAPK inhibitors ○ P21 inhibitors ○ CDK2 inhibitors	○ (Ghosh, 2019) ○ (Romani, 2022) ○ (Aldossary, 2019) ○ (Qi et al., 2019) ○ (Casanova et al., 2021)
Neurotoxicity	○ 36-38% of patients	○ Loss of peripheral limb function ○ Paresthesia ○ Areflexia ○ Loss of proprioception and vibratory sensation ○ Mechanical allodynia ○ Ataxic gait ○ Burning sensations ○ Pain ○ Hemiparesis ○ Status epilepticus ○ coma	○ Treatment discontinuation ○ GSH ○ BNP7787 ○ Vitamine E ○ ORG 2766 ○ Ditiocarb sodium ○ Acetylcysteine ○ Fosfomucin ○ Colestipol ○ Alpha-lipoic acid ○ Diethyldithio-carbamate ○ Nimodipine ○ Oxcarbazepine ○ Retinioic acid	○ (Food and Drug Administration, 2019) ○ (Ghosh, 2019) ○ (Romani, 2022) ○ (Qi et al., 2019) ○ (Trendowski et al., 2019) ○ (Avan et al., 2015)
Ototoxicity	○ 10-93% of patients with greater incidence in children	○ Tinnitus ○ Vertigo ○ Hearing loss in the high frequency range (4000-8000 Hz) ○ Earache	○ STS ○ WR-1065 ○ N-acetyl cysteine ○ Amifostine ○ D-/L-methionine ○ Allopurinol ○ Ebselen ○ Glucocorticoids ○ Copper sulfate ○ Atorvastatin ○ salycilate ○ Noise conditioning ○ Hyperbaric oxygen therapy ○ Therapeutic hypothermia ○ Astaxanthine ○ Curcumin ○ Gaseous H_2 ○ ROSI ○ Alpha-lipoic acid ○ Epigallocatechin-3-gallate ○ Agmatine ○ CPI-455 ○ Rutin ○ Eupatilin ○ RG108 ○ Puerarin ○ Meclofenamic acid ○ Trehalose ○ PRDX1 ○ U0126 ○ NRF2 ○ Polydatin ○ Estradiol ○ D4M ○ Nimodipine ○ Trolox/vitamine E ○ Methylthiobenzoic acid ○ Tiopronin ○ Glutathione ester ○ Dexamethasone ○ Resveratrol ○ Diethyldithiocarbamate ○ Caffeic acid	○ (Food and Drug Administration, 2019) ○ (Ghosh, 2019) ○ (Qi et al., 2019) ○ (Guthrie & Spankovich, 2023) ○ (Y. Li et al., 2023) ○ (Santos, Ferreira, & Santos, 2020) ○ (Fetoni & Astolfi, 2020)

			○ Thioeurea	

Chapter 2. Adopting orphan receptors: group IX/X transition metals as potential ligands of zebrafish Tlr4 homologs

Chapter 2 Preface:

This chapter is in the process of being prepared as a manuscript. At the time of writing, this manuscript is still under revision, and was written by APDF, with editing contributions from WTA. Tracy Lee from the Bhavsar lab was responsible for the in vitro cell culture work. Christie Li generated gRNA sequences as well as PCR primers for crispant validation.

Abstract

Zebrafish (Danio rerio) have gained substantial popularity as a model organism for studying the innate immune system. Their physiology, fecundity, well-characterized development, and similarities to the mammalian immune system help make them an excellent animal model. Pattern recognition receptors (PRRs) play a key role in detecting and initiating a response to damaged cells and invading foreign bodies. One of these receptors, Toll-like receptor 4 (TLR4), is well known for detecting and alerting the host to invading gram-negative bacteria through binding to lipopolysaccharide (LPS). However, there has been a long-standing mystery surrounding the function of Tlr4 homologs within zebrafish, and it's well established that the homologs present in zebrafish have a low sensitivity to LPS. Other ligands, such as transition metals, are known to activate TLR4 through a distinct direct-binding mechanism separate from LPS. We explored the possibility of transition metals acting as ligands to zebrafish Tlr4 homologs, mediating the release of proinflammatory cytokines and leading to cell death. We demonstrate $NiCl_2$, $CoCl_2$, $PtCl_2$, and $PtCl_4$ induce hair cell death in a dose-dependent manner when applied to zebrafish larvae. When zebrafish Tlr4 homologs were transfected into HEK293T cells, nickel can elicit a release of proinflammatory cytokines, while LPS cannot. Finally, treating F0 zebrafish Tlr4 crispant mutants treated with group IX/X metal ions significantly reduced hair cell death without a measurable impact on other aspects of the fish. These findings identify metal ions as adopted ligands for the previously orphaned zebrafish Tlr4 receptors and will improve zebrafish as an animal model for future studies in innate immunology.

2.1 Introduction

TLRs are evolutionarily ancient proteins with origins dating back to more than 700 MYA (million years ago) and can be found in various organisms from corals to humans (Behzadi, Garcia-Perdomo, & Karpinski, 2021; Fitzgerald & Kagan, 2020). Mammalian TLR4 is multifaceted in its function, involving pathogen recognition, cancer pathology, and autoimmune disease (Heine & Zamyatina, 2022). The best-studied function of TLR4 in humans is its ability to detect and bind to microbial pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), and viral glycoproteins, which are essential for the body's inflammatory response to pathogens. The structure of TLR4 is highly conserved between species, however the study of its implications in innate immunity could be furthered with a better understanding of its function in emerging model species, such as zebrafish.

Pattern recognition receptors (PRRs) act as the security cameras of the innate immune system, surveying the host environment for signs of foreign molecules and alarming the host to damage or foreign bodies. TLR4 is a PRR composed of three domains: the extracellular LRR domain (also known as the ectodomain), the transmembrane domain, and the intracellular toll/IL-1 receptor (TIR) domain (Sahoo, 2020; Sameer & Nissar, 2021). This structure is conserved between all vertebrate TLRs and directly contributes to their function (Liu et al., 2020; Sahoo, 2020). Extracellular TLRs (TLR1, 2, 4, 5, 6, and 10) have ectodomains protruding into the extracellular space, making them ideally suited for surveying the environment for antigens present on the surface of organisms and extracellular molecules (Tang et al., 2023). On the other hand, intracellular TLRs (TLR3, 7, 8 and 9) have ectodomains facing the inside of intracellular vesicles, making the detection of intracellular ligands more suited for their location.

Mammalian TLR4's primary ligand, lipopolysaccharide (LPS), is an extracellular compound that begins as a major component of the gram-negative cell wall in bacteria. Signalling through TLR4 using LPS requires multiple different co-receptors acting in concert with one another including: LPS binding protein (LBP), cluster differentiation 14 (CD14), and myeloid differentiation factor 2 (MD-2) (Ryu et al., 2017). Activation of TLR4 leads to downstream signalling and initiation of proinflammatory and type 1 interferon-related gene expression through NF-κB and IRF3, respectively (Figure 2.1). Dysfunction in any of these signalling components has been demonstrated to impede the proper functioning of TLR4 as a bacterial sensor (Ciesielska, Matyjek, & Kwiatkowska, 2021). Recently, the homolog for MD-2 was discovered in zebrafish, prompting Loes et al. to reconsider Tlr4's role in LPS recognition and signalling within zebrafish (2021). Although they found the zebrafish homolog Tlr4ba was capable of being activated by LPS, it's low sensitivity and the lack of other crucial co-receptors in the zebrafish genome suggests that zebrafish Tlr4 primarily recognizes other ligands. Zebrafish MD-2 likely contributes to signalling through LPS, but it also plays a role in signalling through other TLR4 ligands, such as metals.

Metals, such as nickel, that act as ligands of TLR4 were a crucial discovery in determining the involvement of PRRs in mediating metal contact hypersensitivity, a type of allergic contact dermatitis (ACD) (Schmidt et al., 2010). ACD is a common inflammatory skin disease that affects ~20% of the general population and often negatively impacts their quality of life by interfering with their work and social life (Alinaghi et al., 2019; Schram, Warshaw, & Laumann, 2010; Steele, 2022). Two mechanisms are believed to play a role in sensitizing the immune system to nickel (Riedel et al., 2021). One mechanism involves the generation of an adaptive immune response following first contact with the allergen, while the other mechanism is

through the activation of innate immune cells via PRRs, specifically the TLR4 pathway (De Graaf et al., 2023; Riedel et al., 2021; Saito et al., 2016). Like LPS, metal binding to TLR4 leads to the production of proinflammatory cytokines, recruiting immune cells and generating inflammation resulting in itching, tenderness, swelling, and rashes (Figure 2.1)(Katsnelson, 2010; Saito et al., 2016). However, unlike LPS, previous studies have shown that metal binding to TLR4 is through a distinct mechanism that doesn't require the use of co-receptors (Domingo et al., 2023; Peana et al., 2017; Raghavan et al., 2012). They bind directly to the ectodomain of TLR4, leading to its dimerization through a cluster of conserved histidine residues (Oblak, Pohar, & Jerala, 2015; Peana et al., 2017). Instead, Oblak et al. determined MD-2 was only needed to stabilize the TLR4 complex after dimerization and allows for downstream signalling through the MyD88-dependent and -independent pathways (2015).

Zebrafish are a commonly used model organism for studying human disease and have gained recognition as an excellent model for immunological processes (Gomes & Mostowy, 2020; van der Sar et al., 2004). They allow for the examination of cellular interaction at a whole-organism level and possess many of the same innate immune components as humans (Gomes & Mostowy, 2020; Novoa & Figueras, 2012). Zebrafish possess 20 TLRs and express three TLR4 homologs known as Tlr4ba, Tlr4al, and Tlr4bb. Unlike mammalian TLR4, zebrafish Tlr4 does not respond primarily to LPS and lacks the co-receptor CD14, which is required for LPS-induced activation in mammals (Sepulcre et al., 2009; Sullivan et al., 2009). However, their structure remains the same, with all three homologs coding for an extracellular LRR ectodomain, an intermembrane domain and a TIR-containing domain (Figure 2.2 & 2.3)(Vaure & Liu, 2014). Moreover, Loes et al. have recently uncovered the existence of a zebrafish Md-2 homolog that interacts with Tlr4ba, providing evidence of an ancestral interaction between Tlr4 and Md-2

facilitating the detection of an extracellular ligand (2021). In chimeric mouse experiments, their trans-membrane and TIR domains have also shown to function similarly to mammals, capable of eliciting a proinflammatory response after activation (Sullivan et al., 2009). Furthermore, Purcell et al. showed zebrafish and humans possess many of the same intracellular signalling molecules such as MyD88, TIRAP, TRIF, TRAF6, IRF3 and IRF7 (2006).The difference in their ligand specificity appears to lie within the ectodomain, with Tlr4ba, Tlr4al and Tlr4bb showing 35.84, 35.8, and 36.24 % identity to the ectodomain of human TLR4, respectively (Figure 2.3).

The lack of a known primary ligand for zebrafish Tlr4 is puzzling as they appear to have all the components required for sensing an extracellular ligand, yet their true function is still a mystery. Zebrafish appear to lack one of the major co-receptors needed to bind Tlr4 (CD14) and diverge considerably in the structure of their ectodomain, nevertheless, they encode a putative homolog for mammalian MD-2 and can initiate downstream proinflammatory signalling (Loes et al., 2021). Our previous studies have shown zebrafish Tlr4 to be involved in cisplatin-induced ototoxicity (Babolmorad et al., 2021). Morpholino knockdown of tlr4ba and tlr4bb expression mitigated cell death induced by the platinum (II) based chemotherapeutic cisplatin (Babolmorad et al., 2021). Due to these reasons and metals' unique TLR4 activation mechanism, we hypothesize that group IX/X transition metals will initiate zebrafish Tlr4 activation, leading to NFκβ induction and a proinflammatory response. Here, we present $NiCl_2$, $CoCl_2$, $PtCl_2$ and $PtCl_4$ as novel activators and potential novel ligands of zebrafish Toll-like receptor 4 (Tlr4). The findings in this chapter will improve the understanding of Tlr4 function, ultimately improving the utility of zebrafish as an animal model for future studies in innate immunity and disease.

2.2 Methods

1.2.1 Zebrafish Husbandry and Ethics

Zebrafish were kept at the University of Alberta aquatics facility following a 14:10 light/dark cycle in 28.5°C. They were fed twice daily using either trout chow or brine shrimp. All zebrafish were raised, bred, and maintained following the institutional Animal Care and Use Committee approved protocol AUP00000077 which operates under guidelines set by the Canadian Council of Animal Care.

2.2.2 Zebrafish breeding and care

Wildtype (AB strain) zebrafish were bred in a 1.57L tanks using a single male and female or in a 2L tank using a single male and two females. The fish were kept in controlled conditions at 28.5°C overnight. Embryos were then transferred into petri dishes and topped with 25mL of E3 media containing 0.01% methylene blue (Westerfield, 2007). To make the E3 media, a 60X stock solution of embryo media is prepared using 0.29M NaCl, 0.01M KCl, 0.026M $CaCl_2$, 0.001M $MgSO_4$• $7H_2O$. This solution is either diluted down to a 1X solution in Milli-Q water or mixed with 0.01% methylene blue solution in a 5.5:1 ratio before being diluted in Milli-Q water. No more than approximately 100 embryos were placed in each petri dish. Embryos were grown in a 28.5°C incubator and the E3 media was replaced daily.

2.2.3 Metal ion treatment of larval zebrafish

Wildtype (AB strain) zebrafish were grown to either 5- or 6-days post fertilization (dpf) in E3 embryo media with methylene blue and 10-15 larvae were placed in each well of a six-well plate. The larva was then bath treated with either 0, 2.5, 5, 7.5, 10, or 15µM of nickel (II) (Sigma; prod. #654507) or cobalt (II) (Sigma; prod. #255599) chloride hexahydrate or 0, 0.25,

0.5, 0.75, 1, or 1.5µM of platinum (II) (Sigma; prod. #206091) or platinum (IV) (Sigma; prod. #206113) chloride diluted in E3 media with methylene blue for 20 hours at 28°C.

2.2.4 Neuromast quantification assay

The DASPEI quantification assay has been established by many studies, but the one used here was adopted from Babolmorad et al. (2021; Coffin et al., 2009; Harris et al., 2003; Owens et al., 2007; Uribe et al., 2018). Briefly, all wells within the six well plate were washed with embryo media three times before being incubated on an orbital incubator shaker at 130 rpm and 28°C for 15-20 minutes in media containing 0.01% 2-[4-(dimethylamino) styryl]-1-ethylpyridinium iodide (DASPEI, Sigma Aldrich; cat. #3785-01-1) to selectively stain live neuromast hair cells on 6 dpf larva. The wells were washed twice using embryo media and groups of larvae were transferred into individual petri dishes where they were anaesthetized using 4% Tricaine-S (MS-222). After blinding the researcher to treatments, the neuromasts were imaged using a Leica M165 FC dissecting microscope equipped with a GFP-long pass fluorescent filter. Five neuromasts along the posterior lateral line were chosen for scoring consistently throughout experiments (Figure 2.4). Each neuromast was assigned a score ranging from 0-2 based on fluorescent intensity. A score of 2 represented a bright, easily visible neuromast, 1.5 for a minor decline in fluorescence, 1 for a moderate neuromast visibility, 0.5 for a barely visible neuromast, and 0 for no detectable fluorescence. The five neuromast scores were summed for a total score out of 10 for each larva, which represented neuromast viability. A lower score represents less healthy neuromasts, while a high score (near 10) represents healthy neuromasts.

2.2.5 Guide RNA design and injections

Previous work has shown that the biallelic editing ability of clustered regularly interspaced short palindromic repeats (CRISPR)-cas9 technology allows for the generation of F0 "crispant" knockdowns in a target gene with high fidelity (>80%) (Burger et al., 2016; Hoshijima et al., 2019; Jao, Wente, & Chen, 2013; Kroll et al., 2021; Shah et al., 2015). This approach allows for high throughput examination of loss-of-function phenotypes within zebrafish larvae with reduced number of resources and time. Knockdown of TLR4 homologs (generation of *tlr4* "crispants"), used duplexes of trans-activating CRISPR-RNA (tracrRNA) and CRISPR-RNA (crRNA) were made to produce a single guide RNA (sgRNA). The cas9 nuclease binds to the tracrRNA, and is guided to mutate the target gene through the crRNA. crRNAs for *tlr4ba*, *tlr4al,* and *tlr4bb* were designed using the predesigned using Alt-R® CRISPR-Cas9 guide RNA program offered by Integrated DNA Technologies (IDT). The crRNA sequences used can be found in Table 2.1. The final gRNAs for each gene were made by mixing 100µM of the tracrRNA and 100µM of the crRNA, heating them to 95°C for 5 minutes, and diluting them to 25µM using the nuclease-free duplex buffer (IDT; cat. #11-05-01-12). All three gRNAs (25µM) were then combined along with Cas9 protein (25µM) and heated to 37°C for 10 minutes to make a ribonuclease complex (25µM, RNP). The injection solution contained RNP (25µM), 1% dextran tetramethylrhodamine (invitrogen; cat. #D1817), 1M KCl, and RNAse-free water filled to 10µL. Wildtype (AB) zebrafish embryos at the 1 cell stage were injected with either a mock solution containing only the Cas-9 protein (mock injected) or the injection solution containing gRNAs (*tlr4ba/al/bb* crispant). Injected embryos were then incubated at 28.5°C in E3 embryo media.

At 1 dpf dead embryos were removed from the petri dish. At 2 dpf embryos were screened for the injection using a Leica M165 FC dissecting microscope equipped with an mCherry fluorescent filter. Injected embryos showing red fluorescence were sorted and grown to 5dpf at 28.5°C.

2.2.6 Confirming efficacy of tlr4 CRISPR via genotyping

A standard genomic DNA extraction was done on all uninjected, mock and *tlr4ba/al/bb* injected larva that were scored during cisplatin experiments. The extraction was performed by submerging whole larva in 15µL 50µM NaOH and boiling them in the thermocycler at 95°C for 15 minutes, followed by a cooling period of 4°C for 5 minutes. 5µL Tris-HCl was added to neutralize the solution and DNA was stored at 4°C. A PCR was performed on genomic DNA samples using the AllTaq^{TM} Master Mix Kit (Qiagen; cat. #203146) following the manufactures protocol. PCR product concentration was then quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Primers used for the PCR amplification of each zebrafish *tlr4* homolog can be found in Table 2.1. Thermocycling conditions were a 2 minute denaturing period at 95°C, then a specific amplification period for five cycles of 94°C for 15 seconds, 64°C for 15 seconds, and 72°C for 30 seconds, followed by a less-specific amplification for 30 cycles of 94°C for 15 seconds, 54°C for 15 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. Finally, Sanger-sequencing using the same PCR primer sets was performed on the amplified *tlr4ba*, *tlr4al*, and *tlr4bb* genes from 1 uninjected, 1 mock injected and 5 *tlr4* mutant larvae chosen at random.

2.2.7 HEK293T cell transfection and treatment

Human embryonic kidney (HEK) 293T cells (ATCC, catalog (cat) number CRL-3216) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with FBS (10%)

and penicillin-streptomycin (100 μg/mL) at 37°C and 5% CO_2. Cells were seeded in 24-well plates (5 x 104 cells/well) for functional assays. 24 hours after seeding, the HEK293T cells were transfected with an empty vector (pcDNA3-2xHA) or co-transfected with zebrafish Tlr4ba, Tlr4bb (a generous gift from Dr. Victoriano Mulero, Spain)(Sepulcre et al., 2009), or a human TLR4 expression clone (Addgene, cat. #13018) and an empty vector, a mouse MD-2 expression clone (OriGene, cat. #MR225297), or human MD-2 (OriGene, cat. #RC204686). Transfections were done following the manufacturer's protocol using jetPRIME reagent (Polyplus, cat. #CA89129-924) with half the indicated amount of DNA. The media in each well was replaced with fresh media 4 hours post-transfection. 48 hours post-transfection, media was aspirated, and the cells were treated with nickel (II) chloride hexahydrate (Sigma, cat. #379840), or LPS (Invitrogen, cat. #L23351) diluted in fresh media.

2.2.8 Western blot analysis of zebrafish Tlr4 expression

Transfected cells were placed on ice for 5-10 minutes, washed with ice-cold PBS, and returned to ice. Cells were lysed using 200 μL of cold Modified Oncogene Science Lysis Buffer (MOSLB) (10 mM HEPES at pH 7.4, 50 mM Na Pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 uM Na_3VO_4, 1% Triton X-100) containing Roche protease inhibitor cocktail (Sigma, cat. #11697498001) (kindly provided by the Marchant group, UofA MMI) was added to each well and then the cells were placed on a rocker for 15-20 minutes. The wells were rinsed with the lysis buffer and the lysates were collected in pre-chilled microcentrifuge tubes. The lysates were centrifuged at 4°C for 10 minutes at 6000g and then the supernatants were collected in new pre-chilled tubes. Lysates were mixed with 6X Laemmli buffer at a 1:5 (buffer:lysate) ratio and then the samples were loaded without heating in a 10% SDS-PAGE gel. The gel was run at 140 volts and then transferred to a nitrocellulose membrane.

The membrane was blocked with LiCor Intercept (TBS) Blocking Buffer for 1 hour at room temperature, then probed with mouse anti-V5 antibody (1:5000) (Invitrogen, cat. #R96025) overnight at 4^{o}C, and then probed with goat anti-mouse secondary antibody (1:5000) (LiCor, IRDye 800CW) for 1 hour at room temperature. The membrane was washed with TBST and imaged on a LiCor Odyssey.

2.2.9 HEK293T viability and IL-8 secretion

IL-8 cytokine secretion was used as a measure of TLR4 activity per the manufacturer's suggestion for this cell line. Supernatants were collected 24 hours post-treatment and then IL-8 secretion was quantified using commercial human IL-8 ELISA kits (Invitrogen, cat. #88-8086) according to the manufacturer's protocol. IL-8 secretion was normalized to cell viability to account for cell death by nickel. Cell viability was measured using MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (ACROS, cat. #158990010). MTT was diluted to 1 mg/mL in fresh media and added to the cells post-treatment and incubated for 4 hours. Formazan was solubilized in DMSO (dimethyl sulfoxide) (Sigma, cat. #276855) and then the absorbance was measured at 590 nm on a SpectraMAX i3x plate reader (Molecular Devices).

2.2.10 Phylogenetic and synteny analysis of zfTlr4

A phylogenetic reconstruction analysis of TLR4 was performed in MEGA11 as described in Hall et al. (2013; Tamura, Stecher, & Kumar, 2021). Briefly, multiple protein sequences from different vertebrates with e-values <0.001 were collected from NCBI through a BLASTp of zebrafish Tlr4ba in MEGA11. A multiple sequence alignment was performed using MUSCLE under the default options. To determine the most appropriate model for sequence evolution under maximum likelihood (ML) for this data set, we used MEGA's program to find the best model for estimating the tree. The Nearest-Neighbor-interchange (NNI) algorithm was used to generate

initial unrooted tree(s) for the heuristic search, which was estimated using the JJT+F +G +I substitution model with 1000 bootstrap replications (Jones, Taylor, & Thornton, 1992). All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

Synteny analysis was performed using the Genomicus 93.01 available at: https://www.genomicus.bio.ens.psl.eu/genomicus-93.01/cgi-bin/search.pl. The gene tree was rooted at the duplication of Tlr4 in early vertebrates. All Tlr4 sequences are collected from the Ensembl database, with all low-coverage sequences removed from the tree. Species for comparison were then selected for analysis.

2.2.11 Statistical analysis

Individual neuromast scores for each larva were added together to provide a score for each individual animal, and this score was used for subsequent analysis. The mean neuromast viability scores and hair cell count from each group were analyzed using one-way ANOVA with Tukey's multiple comparisons test. All graphs and statistical tests were performed using GraphPad Prism 9. HEK293T normalized IL-8 secretion values were analyzed using an RM two-way ANOVA and Tukey's multiple comparisons test.

2.3 Results

2.3.1 Nickel, cobalt, and platinum induce hair cell death in larval zebrafish neuromasts.

Many genes, including Tlr4, have multiple copies in zebrafish due to the whole genome duplication events that occurred within teleosts. Zebrafish contain three Tlr4 homologs, *tlrba* (ZDB-GENE-040219-8), *tlr4al* (ZDB-GENE-090507-2), and *tlr4bb* (ZDB-GENE-040219-9). Our preliminary synteny analysis, with a wider array of species and complete genomes available, broadly agrees with the analysis performed by Sullivan et al. with respect to the homology

relationships (2009) (Figure 2.5). Furthermore, our phylogenetic analysis also appears to agree with the analysis by Loes et al., in respect to the evolutionary relationship based on sequence similarity (2021). In mammals, nickel is known to induce allergic contact hypersensitivity reactions through the direct binding and activation of TLR4 (Peana et al., 2017). Other group 10 transition metals, such as platinum, have also been shown to activate mammalian TLR4 through a similar mechanism as nickel (Domingo et al., 2023).Previous findings show Tlr4 signalling is highly conserved between mammals and zebrafish, with the lowest conservation area being within the extracellular domain (Sullivan et al., 2009; Zhang et al., 2014). This difference, along with lack of co-receptors, is believed to be responsible for the low sensitivity of zfTlr4zebrafish Tlr4 homologs to LPS and instead may indicate they are more suitable for the direct binding of metal ions.

It has been previously established that a platinum-based chemical is toxic to zebrafish neuromast hair cells in a manner that requires zebrafish Tlr4 homologs (Babolmorad et al., 2021), so we reasoned that hair cell health could be favourable proxy of zebrafish Tlr4 mediating metal toxicity in vivo. Therefore, to determine to the ototoxicity of group 10 transition metals, we first examined their ability to induce hair cell death within the posterior lateral line (pLL) neuromasts. Wildtype zebrafish were exposed to varying concentrations of $NiCl_2$, $CoCl_2$, $PtCl_2$, and $PtCl_4$. DASPEI stained neuromasts showed a dose-dependent decrease in neuromast hair cell viability when treated with nickel (II) and cobalt (II) (Figure 2.6A & B). From these dose response experiments, a working concentration of 10μM (2.38mg/L) $NiCl_2$ and $CoCl_2$ was selected for the following zebrafish experiments based on the low variability in the scores and the significant reduction neuromast viability without complete ablation of the hair cells. In

comparison to untreated larva, neuromast viability scores decreased 5.7-fold and 7.7-fold in larvae treated with 10μM $NiCl_2$ and $CoCl_2$ respectively.

The two platinum salts tested showed approximately a 10-fold increase in toxicity to larval pLL neuromast hair cells compared to nickel and cobalt salts (Figure 2.6C & D). At lower doses, (0μM-1.5μM) $PtCl_2$ and $PtCl_4$ showed mean neuromast viability scores like those seen when larvae were treated with 10-fold higher concentrations of nickel (II) and cobalt (II). From these dose-response curves, we selected a working concentration of 0.75μM for both $PtCl_2$ and $PtCl_4$ due to their near, but not complete ablation of neuromast hair cells. Larvae treated with 0.75μM $PtCl_2$ and $PtCl_4$ showed a 10.7-fold and 2.7-fold decrease in neuromast viability from untreated larvae respectively. $PtCl_2$ showed greater toxicity to pLL neuromast hair cells in comparison to $PtCl_4$ with the mean neuromast viability being 3.8-fold lower at 0.75μM than $PtCl_4$ treated larva at the same concentraiton. Nickel, cobalt, and platinum did not appear to have any overt morphological effects on larva over the course of treatment and their development did not appear impaired (Figure 2.7). Together, these results demonstrate that group X transition metals induce dose-dependent neuromast hair cell death in larval zebrafish.

2.3.2 Zebrafish Tlr4 transfected into HEK293T cells show a modest response to nickel, but no measurable response to LPS.

HEK293T cells do not normally express TLR4 and lack sensitivity to TLR4 agonists, but when transfected with human TLR4 and MD-2, they have been shown to upregulate proinflammatory gene expression in response to known TLR4 agonists (Chow et al., 1999; Medvedev & Vogel, 2003; Yang et al., 2000). After TLR4 stimulation, induction of NF-κB leads to an increase of IL-8 secreted into the media. IL-8 has been established by numerous studies as a marker for TLR4 activation making HEK293T cells an excellent in vitro cell model (Kurt-

Jones et al., 2000; McKee et al., 2021; Oblak, Pohar, & Jerala, 2015; Potnis, Dutta, & Wood, 2013; Quevedo-Diaz et al., 2010; Schmidt et al., 2010). To examine the ability of zebrafish Tlr4 homologs to respond to group 10 transition metals, zebrafish Tlr4ba and Tlr4bb were expressed within the HEK293T cells (confirmed by Western blot, Figure 2.8). IL-8 secretion was measured using ELISA and IL-8 levels were normalized based on cell viability to account for cell death from cisplatin. Treatment with 200μM nickel on cells transfected with Tlr4ba andTlr4bb showed an approximate 2-fold increase from baseline IL-8 secretion with and without MD-2 homologs (Figure 2.9A &B). Cells transfected with human TLR4 showed an approximate 3-fold increase in IL-8 secretion without MD-2 or with mouse Md-2, and an approximate 9-fold increase in IL-8 when transfected with human MD-2 (Figure 2.9C).

Previous studies have shown that nickel binds directly to human TLR4 through conserved histidine residues, inducing dimerization without the requirement of MD-2 (Raghavan et al., 2012; Schmidt et al., 2010). To determine if the presence of MD-2 has an affect on hTLR4 and zebrafish Tlr4 activation, both human or mouse Md-2 homologs were co-transfected with zebrafish Tlr4 homologs or hTLR4. We found the presence of MD-2 was not required for zebrafish Tlr4 and hTLR4 induced IL-8 secretion by nickel as cells transfected with only Tlr4ba, Tlr4bb, or hTLR4 and an empty vector showed a 1.6-fold, 1.7-fold, and 2.9-fold increase in IL-8 secretion, respectively, while cells transfected with the empty vector alone showed no increase from untreated cells (Figure 2.9). Moreover, the addition of either mouse Md-2 or human MD-2 did not have any significant impact on level of IL-8 secretion from cells transfected with zfTrl4ba and zebrafish Tlr4bb (Figure 2.9A & B). It is worth mentioning that Tlr4ba showed a more potent activation in the presence of human MD-2 than mouse Md-2 resulting in a 2-fold increased in IL-8 secretion versus a 1.3-fold increase respectively (Figure 2.9A). The presence of

human MD-2 (hMD2) led to the greatest activation of hTLR4 showing a 9-fold increase in IL-8 secretion, but when co-transfected with the empty vector or mouse Md-2 there was a 3-fold and 3.5-fold increase, respectively (Figure 2.9C). Altogether, this data suggests that nickel-mediated activation of zebrafish Tlr4 or hTLR4 does not require an MD-2 co-receptor, but nickel-induced hTLR4 activation is significantly greater in the presence of human MD-2.

Zebrafish Tlr4 is known not to activated primarily by LPS, therefore LPS was used as a negative control for Tlr4 mediated IL-8 secretion (Figure 2.9A & B). LPS failed to stimulate increased IL-8 secretion in HEK293T cells transfected with either Tlr4ba or Tlr4bb, with and without MD-2. Both zebrafish Tlr4 homologs only show activation mediated by 200µM nickel, while 10ng/mL LPS does not activate Tlr4ba or Tlr4bb. Conversely, LPS is known to be a potent activator of hTLR4 and was used as a positive control in cells transfected with hTLR4. Cells transfected only with the empty vector and treated with 10ng/mL of LPS showed no significant increase in IL-8 secretion from baseline secretion levels (Figure 2.9C). When transfected with hTLR4 alone, there was a significant increase in IL-8 secretion, which was further increased in the presence of human MD-2. Interestingly, cells co-transfected with hTLR4 and mouse Md-2 failed to be activated in the presence of 10ng/mL of LPS, showing selectivity in the co-receptor required to induce IL-8 secretion by LPS.

2.3.3 Zebrafish TLR4 homologs are required for metal toxicity in PLL neuromast hair cells.

Zebrafish F0 genetic mutants (crispants) have recently been used in a variety of studies for examining the effects of targeted gene mutation on disease, behaviour, and development (Bek et al., 2021; Davidson et al., 2021; Lu, Leach, & Gross, 2023; Quick et al., 2021; Trubiroha et al., 2018). They are admired for their effective, accurate, and rapid recapitulation of mutant phenotypes with a mutation rate greater than 80% within the target locus and close resemblance

to their stable mutant counterparts (Hoshijima et al., 2019; Kroll et al., 2021). Here, we used CRISPR-Cas9 gene editing technology to produce a crispant larva where all three zebrafish *tlr4* genes (*tlr4ba/al/bb*) were targeted for mutagenesis. These larvae were then used to establish that the cytotoxic effects on neuromast hair cells from nickel and platinum are mediated through zebrafish Tlr4.

Crispant larva were injected with a Cas9/gRNA ribonuclear complexes (RNPs) targeting a short sequence (~20 nucleotides) early in *tlr4ba*, *tlr4al*, and *tlr4bb* gene homologs for mutation (Figure 2.10). A "mock" injection that contained the Cas9 protein lacking the gRNA duplex was used a control for any effects of the injection process or spontaneous mutations made by the Cas9 protein itself. The mutations, or a lack thereof, were confirmed by taking 10 larvae after metal ion treatment and sequencing the *tlr4* genes after amplifying them with PCR. Sanger Sequencing showed no detectable mutations within the *tlr4* genes of uninjected and mock injected larva, while an unreadable sequence followed the *tlr4ba* and *tlr4bb* genes in crispant larva due to CRISPR mutations leading to disrupted genomic DNA and thus templating of inconsistent PCR products (Figure 2.11-2.13). This confirmed that the *tlr4ba/al/bb* genes were successfully disrupted in crispants, which aligns with our DASPEI scoring experiment results. When examining crispant larval phenotypes, no other noticeable differences were observed compared to uninjected, or mock injected larva (Figure 2.7).

Next, we tested the hypothesis that zebrafish Tlr4 homologs are required for mediating the metal toxicity observed in hair cells. Knockdown of TLR4 in crispants protected hair cells from metal toxicity. After treatment with 10μM $NiCl_2$, 0.75μM $PtCl_2$ and 0.75μM $PtCl_4$, crispants showed an improvement in neuromast hair cell survival (Figure 2.14). The mutations in *tlr4ba* and *tlr4bb* resulted in a near 2-fold increase in mean neuromast viability after treatment

with the platinum compounds, and a 2.5-fold increase in mean neuromast viability for crispants treated with 10μM $NiCl_2$ in comparison to uninjected WT fish. Although these experiments were performed on population of mixed mutants, mutations in the zebrafish *tlr4* homologs allowed for a significant recovery in neuromast viability after metal ion treatment compared to their toxic effects in non-mutant WT larvae. This data along with the in vitro cell culture experiments shows zebrafish Tlr4 homologs are involved in a cell-death response to group 10 transition metals, which act as ligands mediating zebrafish Tlr4ba and Tlr4bb activation and release of proinflammatory cytokines.

2.4 Discussion

This study demonstrates that group IX/X transition metals mediate the activation of Tlr4 homologs in zebrafish, providing insight to the long-standing mystery of TLR4 function in early-branching vertebrates. We showed that bath application of $NiCl_2$, $CoCl_2$, $PtCl_2$, or $PtCl_4$ induced neuromast hair cell death and that this effect required zebrafish Tlr4 homologs. Furthermore, treating cells transfected with zebrafish Tlr4ba or Tlr4bb with nickel, but not LPS, induced the release of IL-8 independently of MD-2. Altogether, this data suggests zebrafish Tlr4 homologs mediate cellular responses to metal ions, which is a response only observed previously in humans and closely related primates.

Exposure to trace metals such as nickel, cobalt, zinc, and copper is common within aquatic environments, and has been previously observed to induce inflammatory responses in aquatic life (Juśkiewicz & Gierszewski, 2022; Singh et al., 2023). Zebrafish have shown metal ions induce expression of proinflammatory cytokines such as *il1β* and *tnfα* as well as Tlr signalling genes *myd88*, *nfkb1a*, and *tlr5b* (Brun et al., 2018; Chen et al., 2019; T. Wang et al., 2015). Moreover, the involvement of Tlrs in mediating these inflammatory responses is

supported by the observation that the MAPK pathway, which is frequently activated during TLR4 signalling, also shows increased gene expression after metal exposure (Chen et al., 2019). Additionally, similar to what is observed in humans following metal-induced TLR activation, contact with metal ions in zebrafish can lead to the development of high concentrations of reactive oxygen species (ROS), leading to further inflammation, disruption of mitchondrial function, and eventually cell death (Jia et al., 2022; T. Wang et al., 2015).

In humans, metals elicit these responses described above through the binding and activation of TLR4 (Babolmorad et al., 2021; Oblak, Pohar, & Jerala, 2015; Rachmawati et al., 2013; Schmidt et al., 2010). TLRs are expressed within the skin of zebrafish (Li et al., 2017), which is the first area contacted by foreign bodies and acts as a primary site for innate immune system activation. In this study we show that during bath application with metal ions, clusters of hair cells on the exterior of zebrafish, called neuromasts, aptly demonstrate the ability of metals to induce cell death (Figure 2.6). This effect can be mitigated by mutations in Tlr4ba/Tlr4bb, resulting in a reduction of metal cytotoxicity (Figure 2.14). This data provides evidence that zebrafish Tlr4 homologs are one of mechanisms involved in the generation of metal-induced cell death.

These findings provide further support to the idea that zebrafish Tlr4 can be activated by metals, and while this suggests an ancient, shared function for vertebrate TLR4 it is noteworthy that not all previous works align exactly with this conclusion. The binding of nickel to human TLR4 has been shown to be directly mediated through two histidine residues within the LRR domain. These residues are conserved between primate species such as chimpanzees and humans but are largely absent in more distantly related vertebrates (Peana et al., 2017; Schmidt et al., 2010) (Figure 2.3). These histidine residues allow for the binding of nickel due to their effective

position within the TLR4 protein, distance in relation to one another, and their nucleophilic imidazole group that interacts strongly with the electrophilic nickel ions. Previously, it has been suggested that due to the lack of these histidine residues, other species apart from primates fail to respond to metal ions through TLR4 (Oblak, Pohar, & Jerala, 2015; Schmidt et al., 2010). Mice have demonstrated nickel hypersensitivity through TLR4 activation, but only when pre-sensitized with LPS as an adjuvant (Sato et al., 2007). Our findings show that cells transfected with zebrafish Tlr4 homologs increase secretion of IL-8 in response to nickel and in the absence of LPS, similar to what is seen in humans (Figure 2.9) (Rachmawati et al., 2013; Schmidt et al., 2010). In contrast to what is known about nickel binding to TLR4 in humans, these findings support the idea that zebrafish Tlr4 can be activated by metals, inducing a proinflammatory response. Furthermore, MD-2 was not required for metal induced activation of zebrafish Tlr4, suggesting a binding mechanism like that observed in humans. Further work is warranted towards understanding which residues in zebrafish Tlr4 mediate metal signalling. Regardless, this implicates zebrafish as a promising animal model for studying metal allergies and provides an in vivo system to examine the complexity of metal-induced contact hypersensitivity reactions and future therapeutic avenues.

Human TLR4 activation can also occur by endogenous molecules released after cell injury called DAMPs. This opens the possibility of two possible mechanisms through which zebrafish Tlr4 activation is observed from metal ion treatment. First, similar to humans, a currently unknown motif within the ectodomain of zebrafish Tlr4 coordinates with metal ions inducing its dimerization and mediating its activation. Second, the metal ions result in the release of DAMPs, which then mediate the activation of zebrafish Tlr4homologs in the response to metals present in the media. DAMP release through the generation of ROS (either through TLR4

or directly by metals) will result in positive feedback loop where activation of TLR4 leads to oxidative stress, leading to the release of DAMPS, which further activates TLR4. Therefore, we can not rule out the possibility of DAMPs as a confounding variable within this study. However, the activation of hTLR4 by some DAMPS such as HMGB1, requires co-receptors such as MD-2, which is not required for the release IL-8 by cells expressing zebrafish Tlr4 (Figure 2.9A & B) (Yang et al., 2015). Furthermore, zebrafish Tlr4 homologs do not have any known endogenous ligands, but examining endogenous activators of zebrafish Tlr4 provides an exciting avenue for future study. Liu et al. showed that peroxiredoxin 1, a widely expressed antioxidant enzyme and DAMP, could interact with Tlr4ba, inducing NF-κB activity and upregulating expression of proinflammatory cytokines (He et al., 2019; 2018). This finding supports some role of DAMPs in zebrafish Tlr4 activation, however more work is required to determine the role DAMPs play in metal induced Tlr4 activaiton in zebrafish.

Apart from histidine, it is known that other residues such as cysteine, aspartic acid, glutamic acid, and methionine contribute to metal ion binding through motifs rich in these amino acids (Kozlowski et al., 2013). Previous studies have established that platinum and platinum containing compounds (such as cisplatin) are capable of directly binding and activating human TLR4 without the requirement of MD-2 (Babolmorad et al., 2021; Domingo et al., 2023). Platinum binding also appears to involve residues disparate from the known nickel binding residues, as activation is still present after mutation of histidine H456 and H458 (Domingo et al., 2023). The platinum compounds tested in this study require lower concentrations to elicit similar levels of neuromast hair cell death as nickel, indicating they are more toxic to zebrafish neuromast hair cells (Figure 2.6). However, no overt toxicity was displayed to the overall health of the zebrafish in response to the platinum compounds (Figure 2.7). In combination with the

crispant mutant experiments that show a significant increase in neuromast viability after Tlr4 mutation, one explanation of these results suggest platinum is a more potent activator of zebrafish Tlr4 homologs compared to nickel, leading to increased inflammation and ROS resulting in more cell death. The stronger activation by platinum may be mediated by other histidine clusters within the zebrafish Tlr4 extracellular domain such as H352, H354 and H361 in Tlr4ba and H445, H450, and H461 in Tlr4bb (Figure 2.3). Moreover, other conserved cysteine, aspartate, and glutamate residues in the LRR region of zebrafish Tlr4 could support platinum activation such as D423, E426, D429, D453, and/or the cysteine cluster C389 and C390. Residues like these within the hypervariable region (proximal region of the extracellular domain) often provide species specific recognition of particular ligands, which in this case could be certain metals like platinum (Vaure & Liu, 2014). These residues act as good candidates to examine more closely for metal binding activity in future studies.

When examining the level of IL-8 secretion between zebrafish Tlr4 and hTLR4, nickel induces greater activation of hTLR4 (Figure 2.9). Across all groups, the greatest increase from baseline IL-8 secretion was a 9-fold increase induced in cells co-transfected with hTLR4 + human MD-2 and treated with nickel (Figure 2.9C). On the other hand, the greatest increase in IL-8 secretion from the zebrafish Tlr4 transfection experiments occurred in cells co-transfected with Tlr4ba + human MD-2, with a 2-fold increase in IL-8 from baseline (Figure 2.9.A&B). The difference in maximal activation levels may be due to zebrafish Tlr4 lacking it's recently identified endogenous MD-2 co-receptor (Ly96) leading to incomplete signalling (Loes et al., 2021). When comparing between species, we see that the levels of IL-8 secretion from nickel treated cells co-transfected with either hTLR4 or zebrafish Tlr4 and an empty vector or mouse Md-2 are similar (Figure 2.9). A previous study by Raghavan et al. showed that, although still

present, activation of hTLR4 without human MD-2 by nickel and cobalt is significantly reduced (2012) (Figure 2.9). This suggests that when Tlr4 receptors are alone, or lacking their endogenous co-receptor, the activation of the receptor is stunted from its full potential. Our data supports this finding, where we observed hTLR4 subject to nickel treatment in the presence of mouse Md-2 had a significantly reduced response in comparison to when the cells were co-transfected with human MD-2, its endogenous co-receptor (Figure 2.9C). Similarly, certain ligands like taxol, LPS from *Rhodobacter sphaeroides*, and tetraacylated lipid IVa elicit different responses from TLR4 depending on the species of TLR4/MD-2 complex present (Ohto et al., 2012; Werling et al., 2009). Therefore, it is possible that residues within the hypervariable variable region of zebrafish Tlr4 and the endogenous zebrafish MD-2 homolog would assist in providing crucial interactions required to promote a more potent response to metal ions.

The ability of zebrafish Tlr4, particularly Tlr4ba, to respond to LPS was recently re-visited by Loes et al. after their insightful observation of an MD-2 homolog present within the zebrafish genome (2021). LPS was shown to be capable of activating zebrafish Tlr4ba in the presence of MD-2, however, there are two caveats: it requires mammalian Cd14, which currently has no known homolog within zebrafish, and the concentrations required for activation of zebrafish Tlr4 was also considerably higher (ug/mL) than the biologically relevant concentrations of LPS (0-10ng/mL) required for TLR4 activation (Guo et al., 2013). On the other hand, dimerization by metal ions is through a direct binding mechanism and excludes the prerequisite of CD14 for activation. The presence of Tlr4 homologs and an MD-2 homolog within zebrafish along with the lack sensitivity to LPS provides a logical basis for our hypothesis that zebrafish Tlr4 utilizes group IX/X metals for ligands. The most recent hypotheses for how zebrafish Tlr4 fits in to the evolution of this receptor suggests the *tlr4* gene along with *ly96* (MD-

2) arose in a common ancestor before divergence of lobe-finned and ray-finned fish (Loes et al., 2021). The lack CD14 and LPS sensitivity in most fish species, but the presence of MD-2, further supports the notion that Tlr4's purpose originally included the binding of ligands that do not require co-receptors, such as metals.

In summary we present data in support of nickel and platinum acting as novel ligands for zebrafish Tlr4, helping discern the mystery surrounding its function. We demonstrated that through genetic mutation of Tlr4, zebrafish become significantly less sensitive to metal cytotoxicity and their presence in cells increased the inflammatory response to metals. These findings suggest a previously unknown function of zebrafish Tlr4 and that they may be used to sense for metals in their environment, resulting in toxicity and inflammation observed in zebrafish. Further understanding the function of zebrafish Tlr4 and the involvement of metal ions will facilitate better utilization of this model organism in the study of innate immunity and disease, particularly metal contact hypersensitivity.

2.5 Figures and Tables

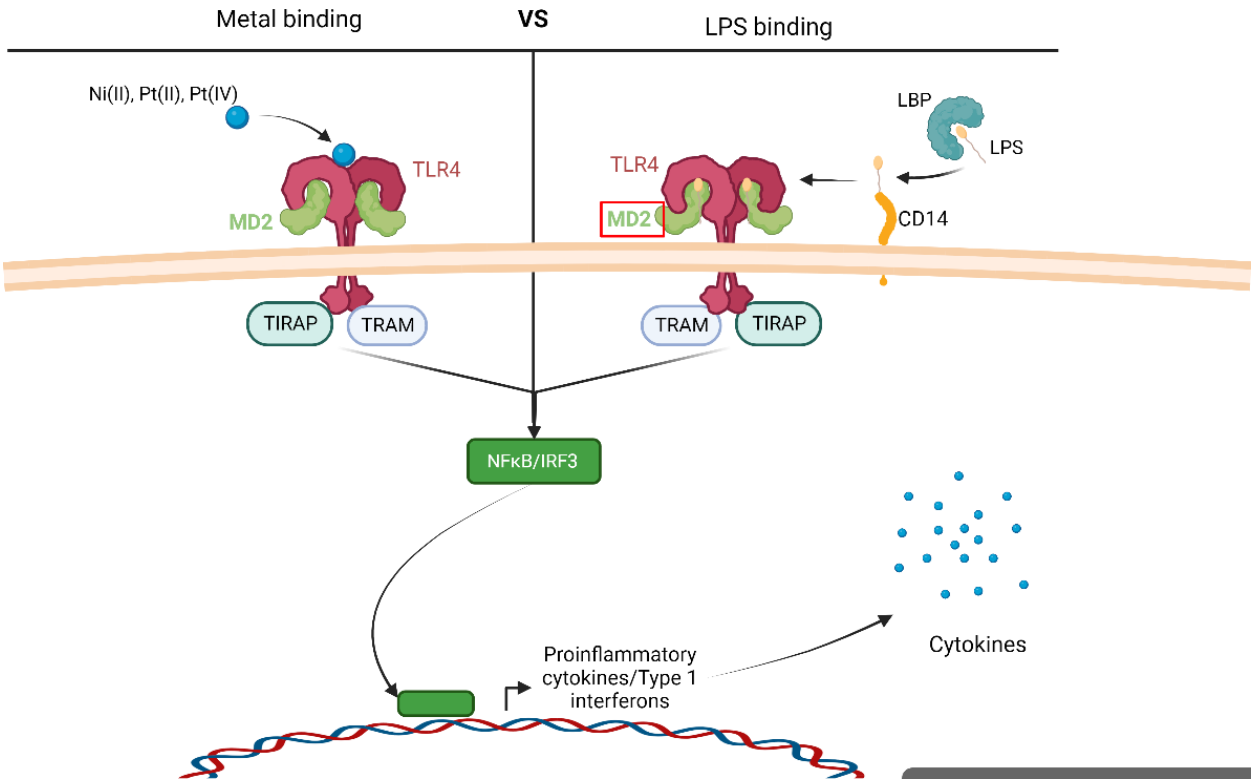
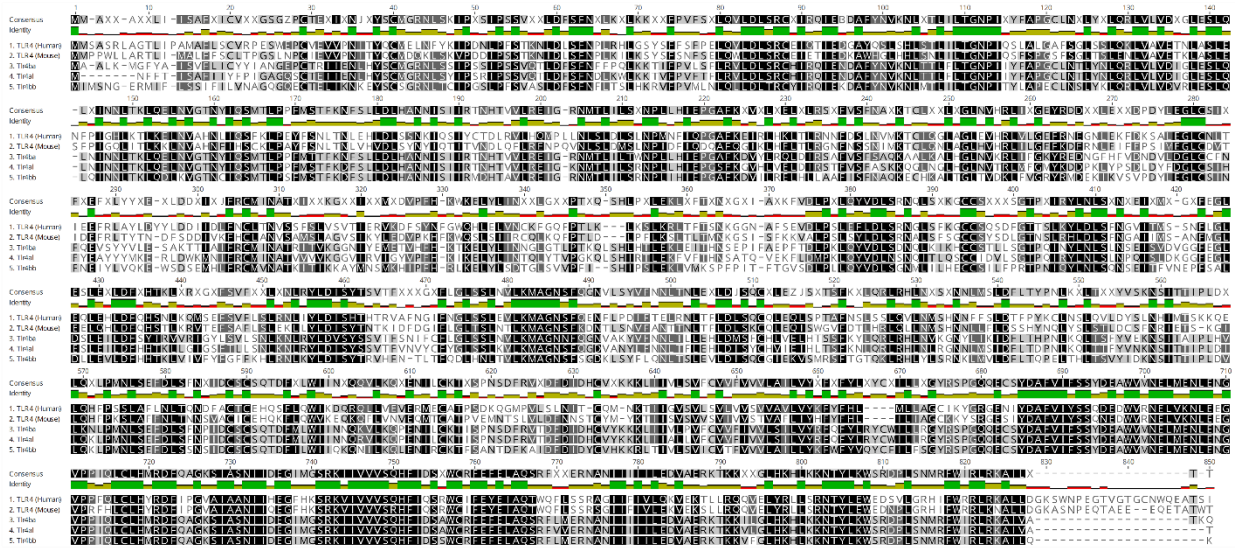


Figure 2. 1 Metal ions bind directly to TLR4 versus co-receptor binding with LPS. Group IX/X transition metals bind directly to key residues in the ectodomain of toll-like receptor 4 (TLR4). This stimulates nuclear factor-κβ (NF- κβ), leading to the release of type 1 interferons and proinflammatory cytokines. LPS binds to the LPS Binding Protein (LBP) and is transferred to the co-receptor cluster of differentiation 14 (CD14). CD14 facilitates the binding of LPS to myeloid differentiation factor 2 (MD-2)/TLR4 signalling complex, which then dimerizes and follows a similar signalling cascade as described above. Created with BioRender.

A

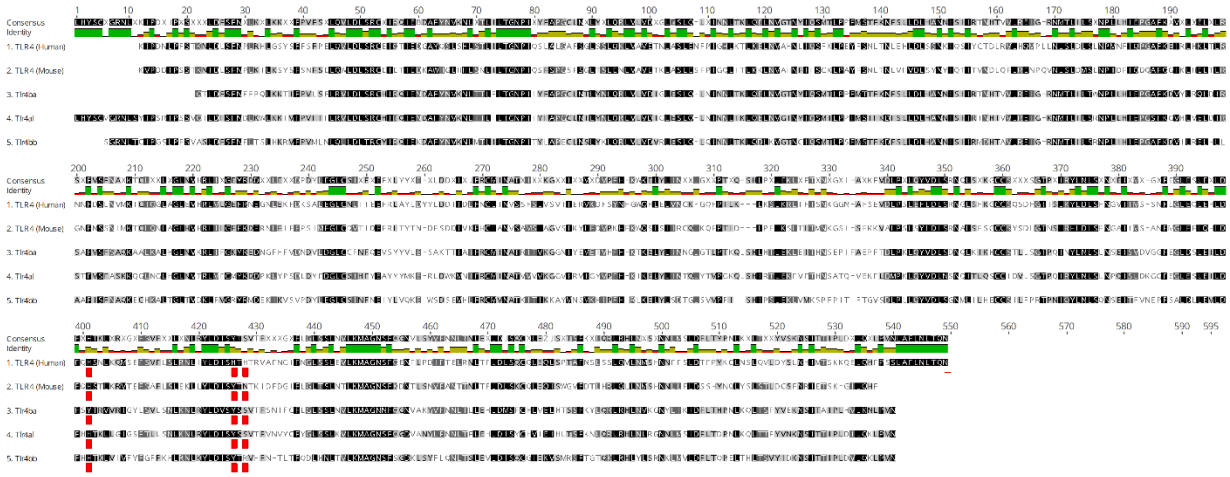


B

	Tlr4ba	Tlr4al	Tlr4bb	TLR4 (Hum...	TLR4 (Mou...
Tlr4ba		79.37%	64.00%	37.29%	35.70%
Tlr4al	79.37%		65.09%	36.63%	36.33%
Tlr4bb	64.00%	65.09%		36.56%	34.36%
TLR4 (Human)	37.29%	36.63%	36.56%		66.43%
TLR4 (Mouse)	35.70%	36.33%	34.36%	66.43%	

Figure 2. 2 Multiple sequence alignment and precent identity matrix for TLR4 (full protein) between zebrafish, mice, and humans. A) The comparison between sequences shows the homology and conservation of residues mainly in the latter third of the protein sequence. Similar residues (100%) are highlighted in black, partially similar residues are highlighted in dark grey (80%-100%) and light grey (60%-80%). The coloured histogram shows the mean pairwise precent identity for each column of the alignment, where green represents 100%, yellow represents >30%, and red represents <30%. B) Table shows the percent sequence identity between mammalian TLR4 (mouse and human) is >60%, while the precent identity between mammalian and zebrafish Tlr4 homologs <40%. Alignments, similarity, and the percent identity matrix were made and calculated using Geneious Prime.

A



B

	Tlr4ba	Tlr4al	Tlr4bb	TLR4 (Hum...	TLR4 (Mou...
Tlr4ba		72.62%	56.50%	35.84%	34.95%
Tlr4al	72.62%		57.92%	35.80%	36.26%
Tlr4bb	56.50%	57.92%		36.24%	33.27%
TLR4 (Human)	35.84%	35.80%	36.24%		62.57%
TLR4 (Mouse)	34.95%	36.26%	33.27%	62.57%	

C

D

	Tlr4ba	Tlr4al	Tlr4bb	TLR4 (Hum...	TLR4 (Mou...
Tlr4ba		97.79%	97.79%	54.41%	52.21%
Tlr4al	97.79%		97.18%	55.63%	52.11%
Tlr4bb	97.79%	97.18%		55.63%	52.11%
TLR4 (Human)	54.41%	55.63%	55.63%		91.55%
TLR4 (Mouse)	52.21%	52.11%	52.11%	91.55%	

Figure 2. 3 Multiple sequence alignment and precent identity matrix for TLR4 extracellular LRR domain and intracellular TIR domain between human, mouse, and zebrafish. The comparison between sequences shows the homology and conservation of residues is ~20% greater within the intracellular TIR domain compared with the extracellular LRR domain. Similar residues (100%) are highlighted in black, partially similar residues are highlighted in dark grey (80%-100%) and light grey (60%-80%). The coloured histogram shows

the mean pairwise precent identity for each column of the alignment, where green represents 100%, yellow represents >30%, and red represents <30%. The known human nickel binding residues are underlined in red within A. The nickel binding residues 431 and 456 show 100% similarity between humans, mice, and zebrafish, as well as partial identity (>30%) and consist of either histidine or tyrosine residues. The last known nickel binding residue (458) shows low similarity and identity between all sequence and is either a histidine, asparagine, serine, or arginine residue. The table below the alignments show the percent sequence identity for the entire sequence alignment between human TLR4, mouse TLR4, zebrafish Tlr4ba, Tlr4al, and Tlr4bb. Alignments, similarity, and the percent identity matrix were made and calculated using Geneious Prime.

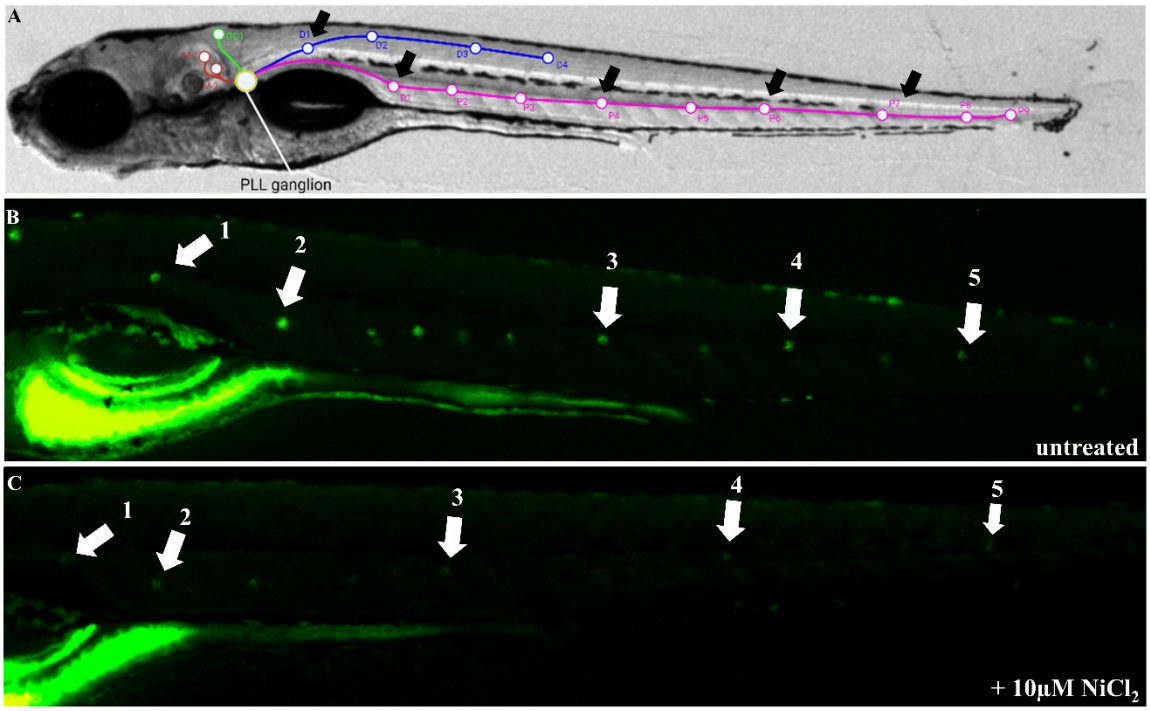


Figure 2. 4 Depiction of the zebrafish posterior lateral line and neuromasts in 7dpf zebrafish larvae. A) Location of posterior lateral line neuromasts drawn on a bright field image of a 7dpf larva. Black arrows point to the location of the five stereotypically developed neuromasts consistently scored between experiments B) DASPEI stained untreated larva with visible neuromasts. White arrows point towards the same five neuromasts as shown in A. C) DASPEI stained larva treated with 10µM nickel (II) chloride hexahydrate, showing the lack of fluorescence from neuromasts due to a hair cell death.

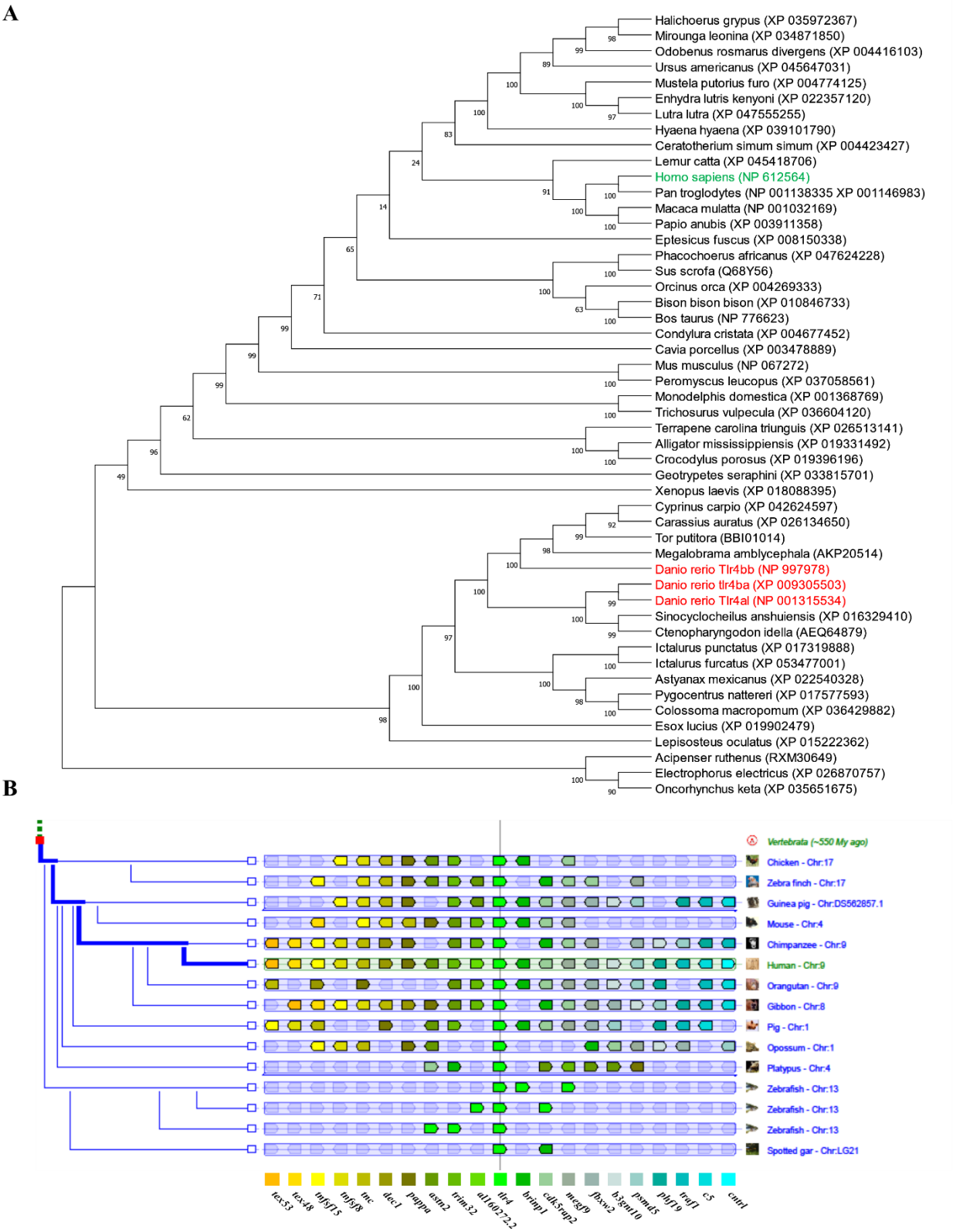

A
Halichoerus grypus (XP 035972367)
Mirounga leonina (XP 034871850)
Odobenus rosmarus divergens (XP 004416103)
Ursus americanus (XP 045647031)
Mustela putorius furo (XP 004774125)
Enhydra lutris kenyoni (XP 022357120)
Lutra lutra (XP 047555255)
Hyaena hyaena (XP 039101790)
Ceratotherium simum simum (XP 004423427)
Lemur catta (XP 045418706)
Homo sapiens (NP 612564)
Pan troglodytes (NP 001138335 XP 001146983)
Macaca mulatta (NP 001032169)
Papio anubis (XP 003911358)
Eptesicus fuscus (XP 008150338)
Phacochoerus africanus (XP 047624228)
Sus scrofa (Q68Y56)
Orcinus orca (XP 004269333)
Bison bison bison (XP 010846733)
Bos taurus (NP 776623)
Condylura cristata (XP 004677452)
Cavia porcellus (XP 003478889)
Mus musculus (NP 067272)
Peromyscus leucopus (XP 037058561)
Monodelphis domestica (XP 001368769)
Trichosurus vulpecula (XP 036604120)
Terrapene carolina triunguis (XP 026513141)
Alligator mississippiensis (XP 019331492)
Crocodylus porosus (XP 019396196)
Geotrypetes seraphini (XP 033815701)
Xenopus laevis (XP 018088395)
Cyprinus carpio (XP 042624597)
Carassius auratus (XP 026134650)
Tor putitora (BBI01014)
Megalobrama amblycephala (AKP20514)
Danio rerio Tlr4bb (NP 997978)
Danio rerio tlr4ba (XP 009305503)
Danio rerio Tlr4al (NP 001315534)
Sinocyclocheilus anshuiensis (XP 016329410)
Ctenopharyngodon idella (AEQ64879)
Ictalurus punctatus (XP 017319888)
Ictalurus furcatus (XP 053477001)
Astyanax mexicanus (XP 022540328)
Pygocentrus nattereri (XP 017577593)
Colossoma macropomum (XP 036429882)
Esox lucius (XP 019902479)
Lepisosteus oculatus (XP 015222362)
Acipenser ruthenus (RXM30649)
Electrophorus electricus (XP 026870757)
Oncorhynchus keta (XP 035651675)
B
Vertebrata (~550 My ago)
Chicken - Chr:17
Zebra finch - Chr:17
Guinea pig - Chr:DS562857.1
Mouse - Chr:4
Chimpanzee - Chr:9
Human - Chr:9
Orangutan - Chr:9
Gibbon - Chr:8
Pig - Chr:1
Opossum - Chr:1
Platypus - Chr:4
Zebrafish - Chr:13
Zebrafish - Chr:13
Zebrafish - Chr:13
Spotted gar - Chr:LG21
tex33
tex48
tnfsf15
tnfsf8
tnc
dec1
pappa
asm2
trim32
al160272.2
tlr4
brinp1
cdk5rap2
mcgf9
jhdm2
b3gnt10
psmd5
phf19
traf1
c5
cntrl

Figure 2. 5 Phylogenetic and synteny analysis of TLR4 sequences between species. A) Maximum likelihood unrooted phylogenetic tree of 48 TLR4 protein sequences. The percentage of trees in which the associated taxa clustered together is shown below the branches. The human TLR4 sequence is highlighted in green, while zebrafish Tlr4 sequences are highlighted in red. B) Synteny analysis examining the conservation of 20 genes surrounding human *tlr4* between 13 vertebrate species. Primates (humans, chimpanzee, orangutan, gibbon) share a high level of chromosome structure with humans with many orthologs located in a similar location relative to *tlr4*. The conservation of chromosome structure decreases with earlier divergence of species. *tlr4* from different vertebrate species are aligned down the middle column. Each row shows a short area of chromosomal structure surrounding *tlr4* in different species. The blocks indicate the location of genes in different species that share homology with their human counterpart. Each colour corresponds to an ortholog of a gene, with the human gene name and its corresponding colour denoted at the bottom. The gene tree is shown on the left while organism name and the chromosomal location of the gene sets are indicated on the right. Multiple gene alignment for synteny analysis was made using Genomicus 93.01. Evolutionary analyses were conducted in MEGA11 (Tamura, Stecher, & Kumar, 2021).

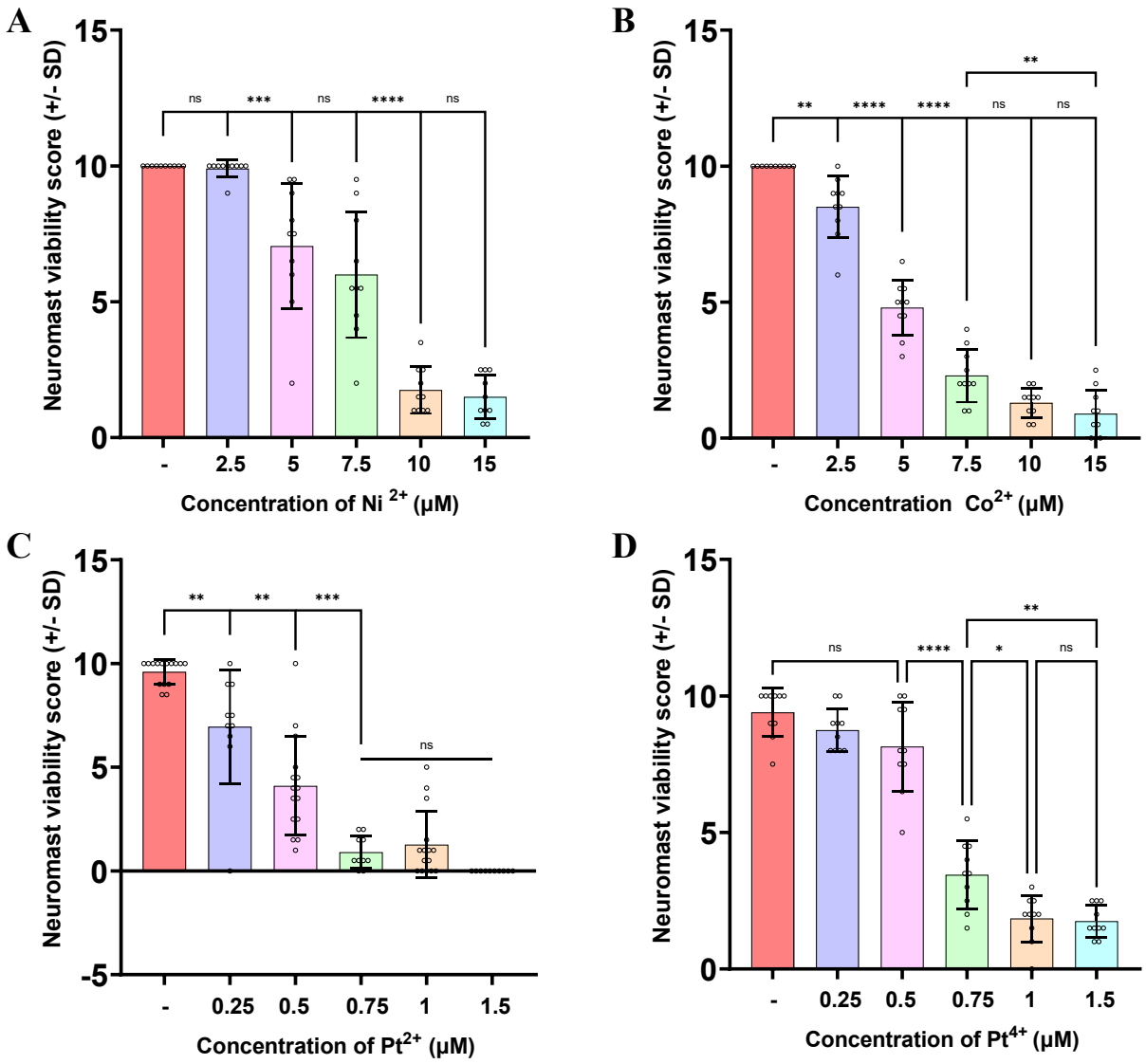


Figure 2. 6 Nickel (II) chloride, cobalt (II) chloride, platinum (II) chloride, and platinum (IV) chloride induce PLL hair cell death in a dose dependent manner within 6-7dpf zebrafish. As the concentration of the $NiCl_2$ (A), $CoCl_2$ (B), $PtCl_2$ (C), and $PtCl_4$ (D) in the media increased, the intensity of DASPEI fluorescence decreased, indicating a decrease in neuromast hair cell viability. $NiCl_2$ and $CoCl_2$ are less toxic to neuromast hair cells as the concentration of $NiCl_2$ and $CoCl_2$ required for similar levels of neuromast hair cell death to occur

is 10-fold that of the platinum salts. N = 10 wildtype larva for all treatment groups. Bars represent the mean neuromast viability score of all larvae. ****$p<0.0001$, ***$p<0.0002$, **$p<0.0021$, *$p<0.0332$, ns = not significant by one way ANOVA and Tukey's multiple comparison test.

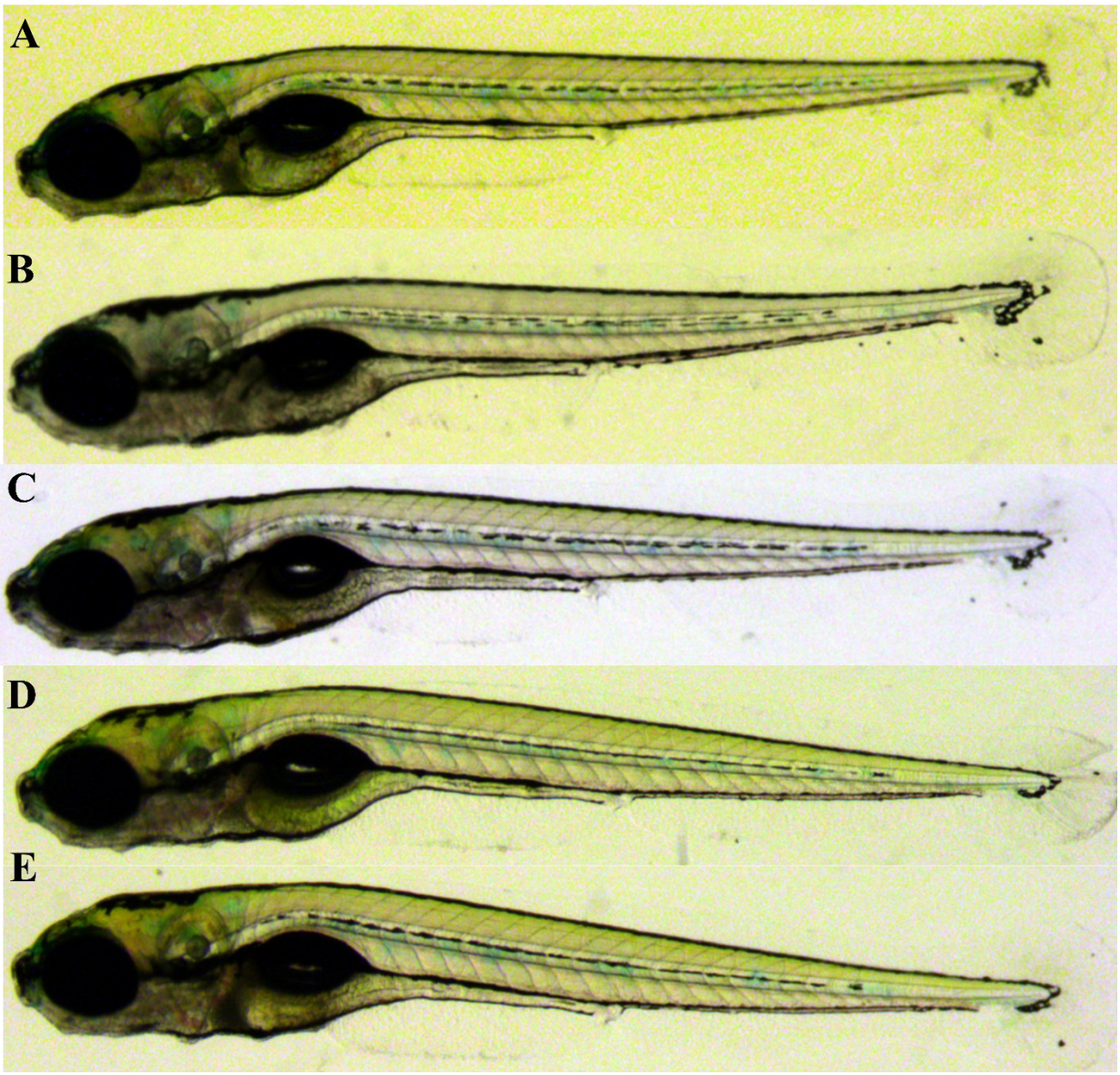


Figure 2. 7 Tlr4 crispant fish treated with nickel (II) and platinum (IV). A-C) 6-7dpf mock injected and Tlr4 crispant larva treated with either $PtCl_4$ or $NiCl_2$ show no apparent morphological change in comparison to an uninjected untreated larva. A) Uninjected, untreated larva. B) Mock injected larva treated with 7.5µM $PtCl_4$. C) Tlr4 crispant treated with 7.5µM $PtCl_4$. D) Mock injected larva treated with 10µM $NiCl_2$. E) Tlr4 crispant larva treated with 10µM $NiCl_2$.

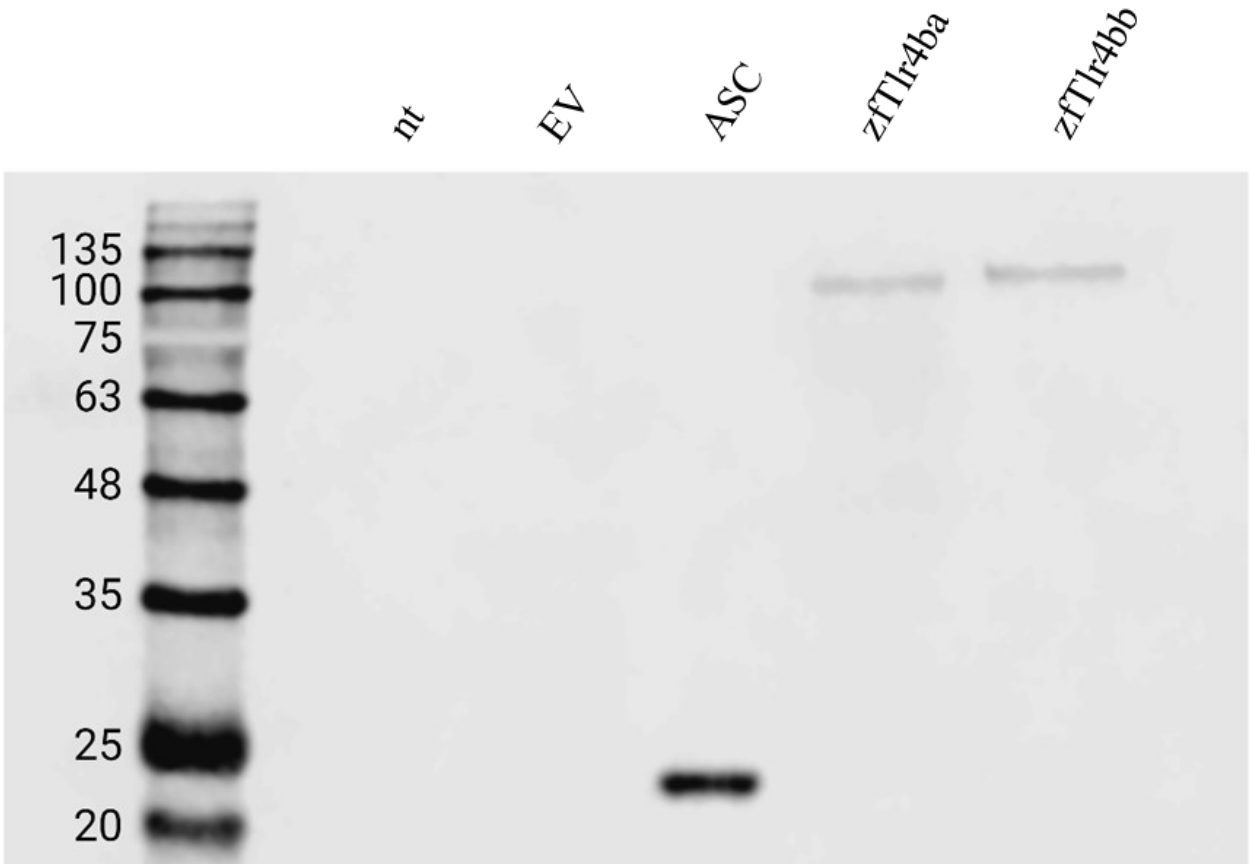


Figure 2. 8 Western blot showing expression of zebrafish Tlr4ba and Tlr4bb in HEK293T cells. Zebrafish *tlr4ba* and *tlr4bb* were transfected individually into HEK293T cells and expressed using the SV40 promoter. Untransfected HEK293T cells and those transfected with an empty vector (EV) show no expression of a Tlr4 protein, but those transfected with the *tlr4* recombinant plasmids show expression of a protein at approximate size of Tlr4ba (~86 kDa) and Tlrbb (~95kDa) respectively. Additionally, zebrafish Tlr4 proteins were tagged with a 5' V5/His_6 sequence for detection using mouse anti-V5 antibodies. Expression of ASC was used as a positive control. The ladder (left) shows proteins of known sizes in kDa. nt = no transfection, EV = empty vector, ASC = apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD), zfTlr4ba = zebrafish Tlr4ba, zfTlr4bb = zebrafish Tlr4bb.

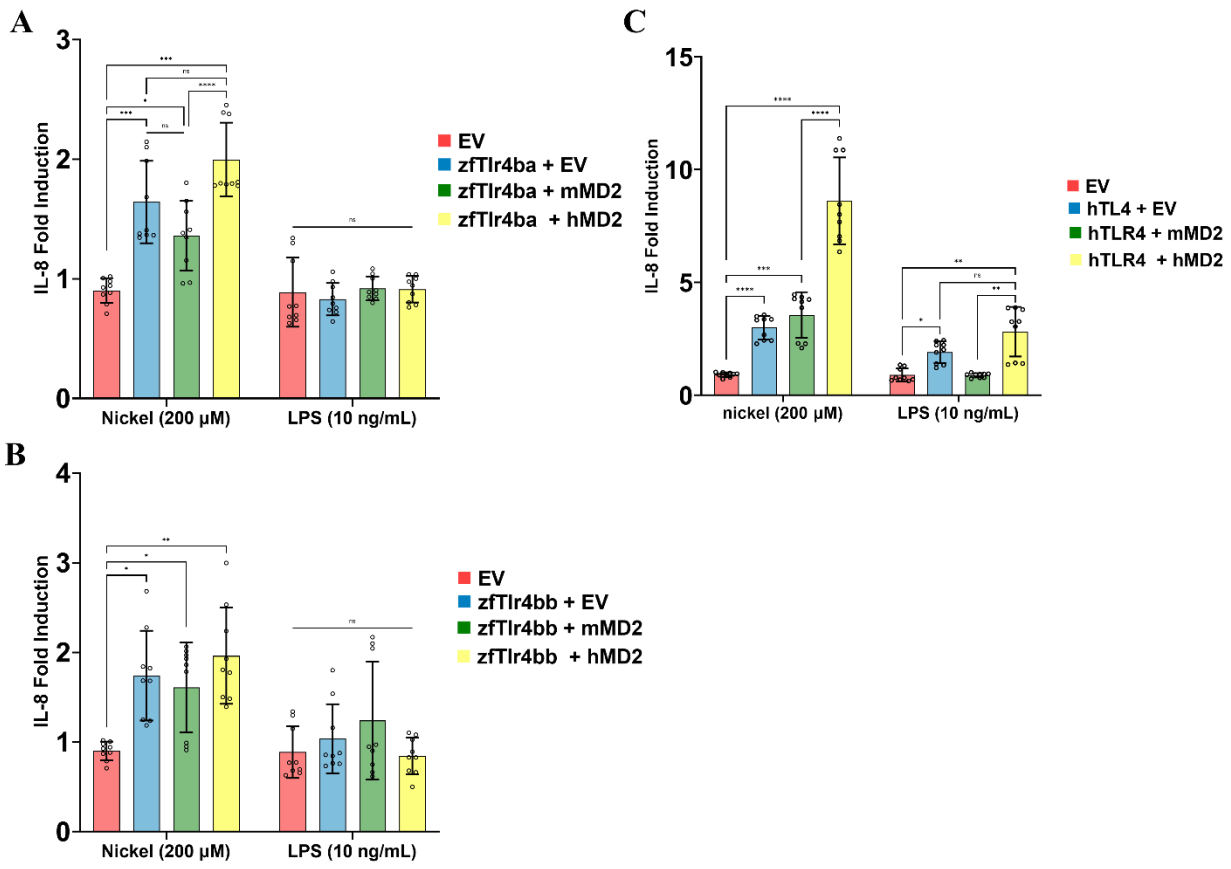


Figure 2. 9 Nickel activates zebrafish Tlr4ba and Tlr4bb in HEK293T cells while LPS does not. The TLR4 signalling cascade is well conserved between zebrafish and mammals. Stimulation of TLR4 by LPS in mammals results in a release of a range of proinflammatory cytokines and type 1 interferons. Cells transfected with Tlr4ba (A), Tlr4bb (B), or human TLR4 (hTLR4; C) and an empty vector, mouse (mMD2) or human (hMD2) MD-2 show an increase in IL-8 secretion (a common measure of TLR4 activation) after treatment with nickel (II) chloride hexahydrate in comparison to cells transfected with the empty vector alone. A-B) HEK293 cells lacking mammalian TLR4 but transfected with Tlr4ba and Tlr4bb show increased secretion of IL-8 when treated with 200µM nickel (II) chloride hexahydrate, but do not when treated with 10ng/mL of LPS. Stimulation of IL-8 release by Tlr4ba (A) and Tlr4bb (B) does not require MD-

2. A) The Tlr4ba-hMD-2 complex allows for increased IL-8 secretion in the presence of Ni^{2+} in comparison to the mMD-2 homolog. B) zfTLr4bb does not require or show a preference for either MD-2 homologs when inducing IL-8 secretion in the presence Ni^{2+}. C) hTLR4 induces IL-8 secretion both in the presence of Ni^{2+} and LPS but does not when transfected with the empty vector alone. hTLR4 induces IL-8 secretion after nickel (II) chloride hexahydrate treatment with or without MD-2 but is significantly greater when transfected with hMD-2. LPS induces IL-8 secretion with and without hMD-2 but does not when mMD-2 is present. The secretion of IL-8 is significantly greater in cells transfected with hMD-2 in comparison to cells transfected with the empty vector alone. IL-8 fold induction was calculated based on the baseline cell secretion of IL-8 without treatment. N = three independent experiments for all treatments. ****$p<0.0001$, ***$p<0.0002$, **$p<0.0021$, *$p<0.0332$, ns = not significant by two-way RM ANOVA and Šídák's multiple comparisons test. This data was generated and provided with permission from Tracy Lee.

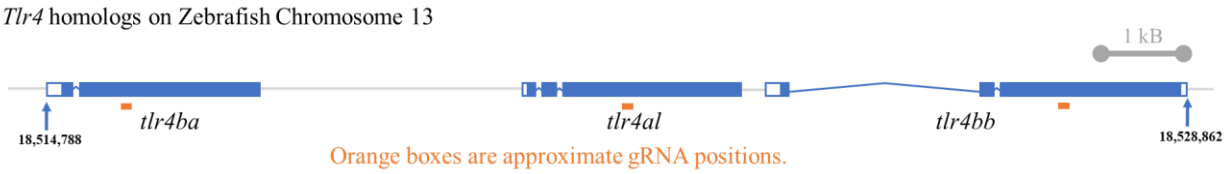


Figure 2. 10 Illustration representing the 3 *tlr4* homologs on zebrafish chromosome 13 and their approximate gRNA positions. The three zebrafish *tlr4* homologs are found in succession to one another. Arrows point to the initial base pair position on the chromosome. White boxes represent untranslated regions, blue boxes show exons, while blue lines show introns. Grey lines represent the intergenic region connecting the homologs.

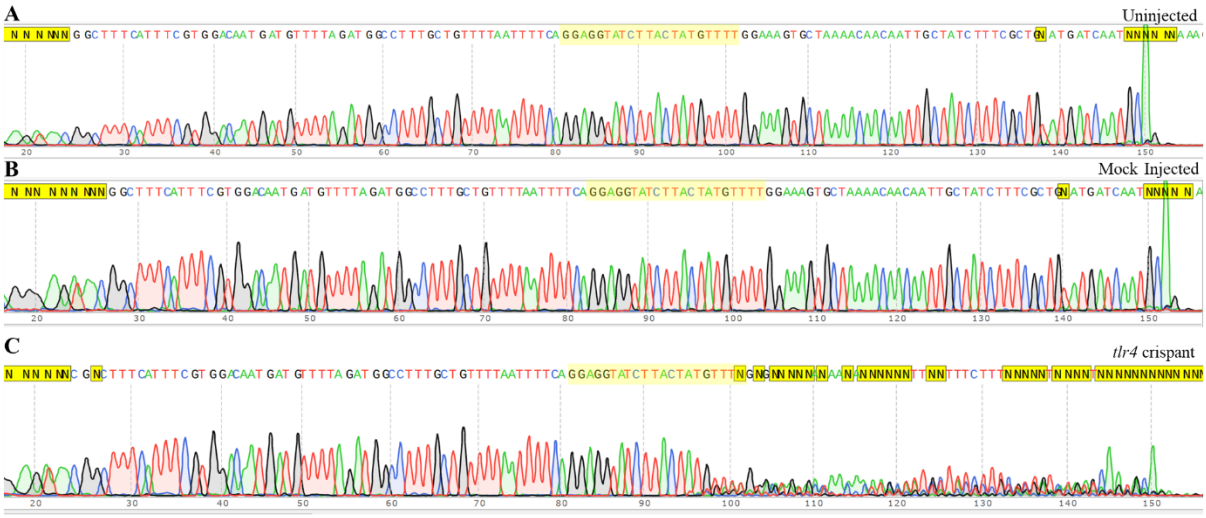


Figure 2. 11 Sanger Sequencing of tlr4ba crispant DNA. The *tlr4ba* gene was amplified from the genomic DNA of individual larvae via PCR using the tlr4ba forward primer. Uninjected larva (A) and mock injected larva (B) DNA in A & B shows an unmutated *tlr4ba* sequence, where consistent DNA in all cells produces a coherent chromatogram throughout. C) Chromatograms from *tlr4* cripants show clear sequence chromatograms where the DNA is homozygous and consistent throughout the individual (left side), but the chromatogram becomes unreadable near the gRNA binding site (highlighted in light yellow) because a mixture of various mutant DNA sequences now exists among the larva's cells. Larva were injected at the one cell stage with either a gRNA sequence targeting the three zebrafish tlr4ba homolog or Cas9 protein alone (mock).

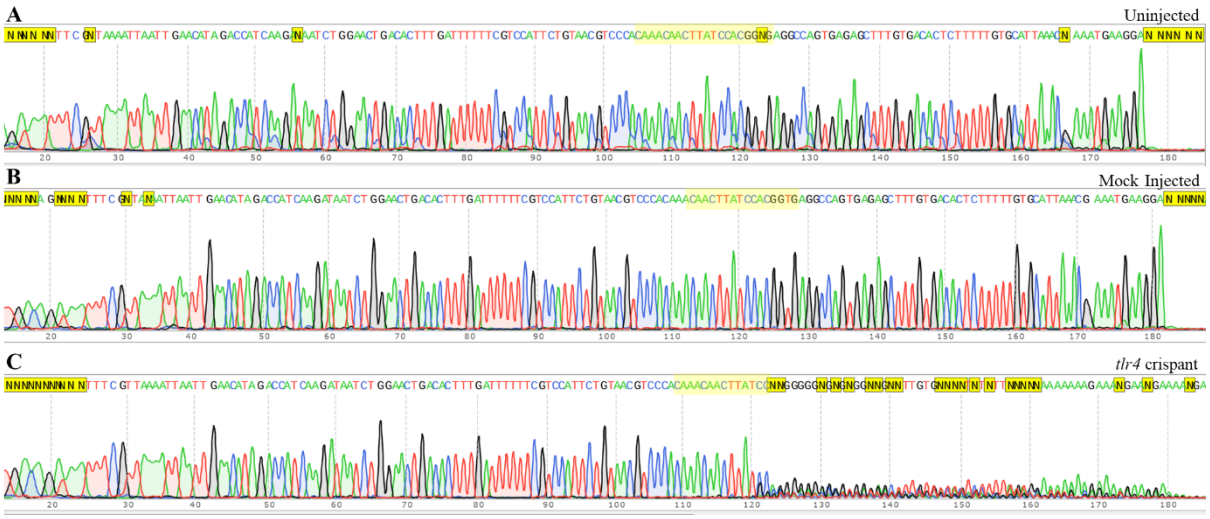


Figure 2. 12 Sanger Sequencing of tlr4bb crispant DNA. The *tlr4bb* gene was amplified from the genomic DNA of individual larvae via PCR using the *tlr4bb* reverse primer. Uninjected larva (D) and mock injected larva (E) DNA in A & B shows an unmutated tlr4bb sequence, where consistent DNA in all cells produces a coherent chromatogram throughout. F) Chromatograms from tlr4 cripants show clear sequence chromatograms where the DNA is homozygous and consistent throughout the individual (left side), but the chromatogram becomes unreadable near the gRNA binding site (highlighted in light yellow) because a mixture of various mutant DNA sequences now exists among the larva's cells. Larva were injected at the one cell stage with either a gRNA sequence targeting the three zebrafish tlr4bb homolog or Cas9 protein alone (mock).

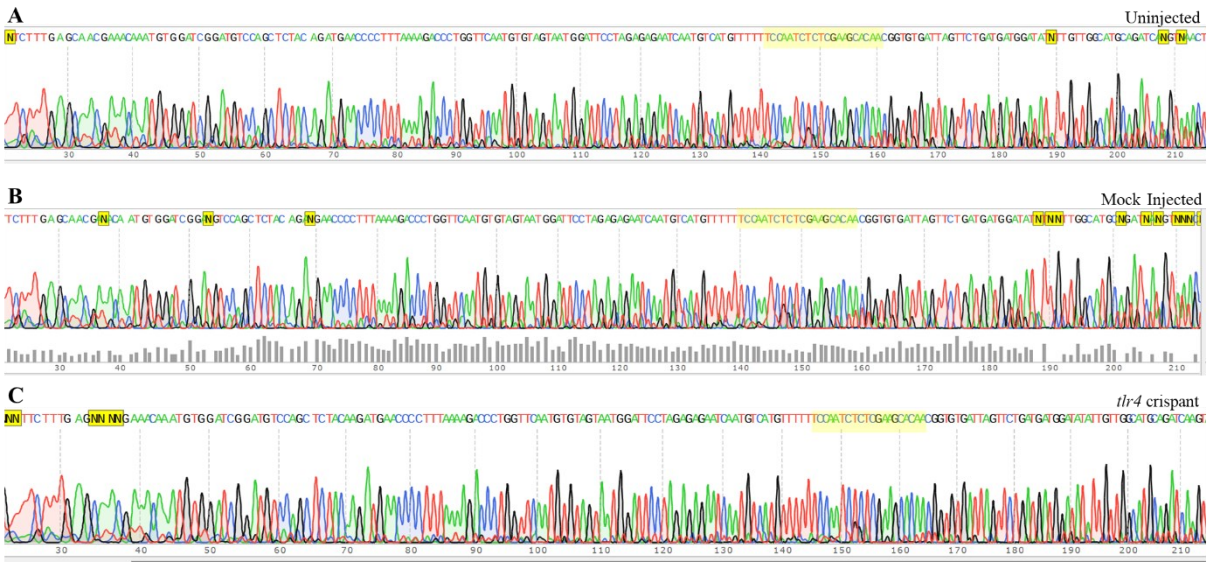


Figure 2. 13 Sanger Sequencing of tlr4al crispant DNA. The *tlr4al* gene was amplified from the genomic DNA of individual larvae via PCR using the *tlr4al* reverse primer. Uninjected larva (G) and mock injected larva (H) DNA in G & H shows an unmutated *tlr4al* sequence, where consistent DNA in all larval cells produces a coherent chromatogram throughout. I) Chromatograms from *tlr4* cripants show clear sequence chromatograms where the DNA is homozygous and consistent throughout the individual even following the gRNA target sequence (highlighted in light yellow).

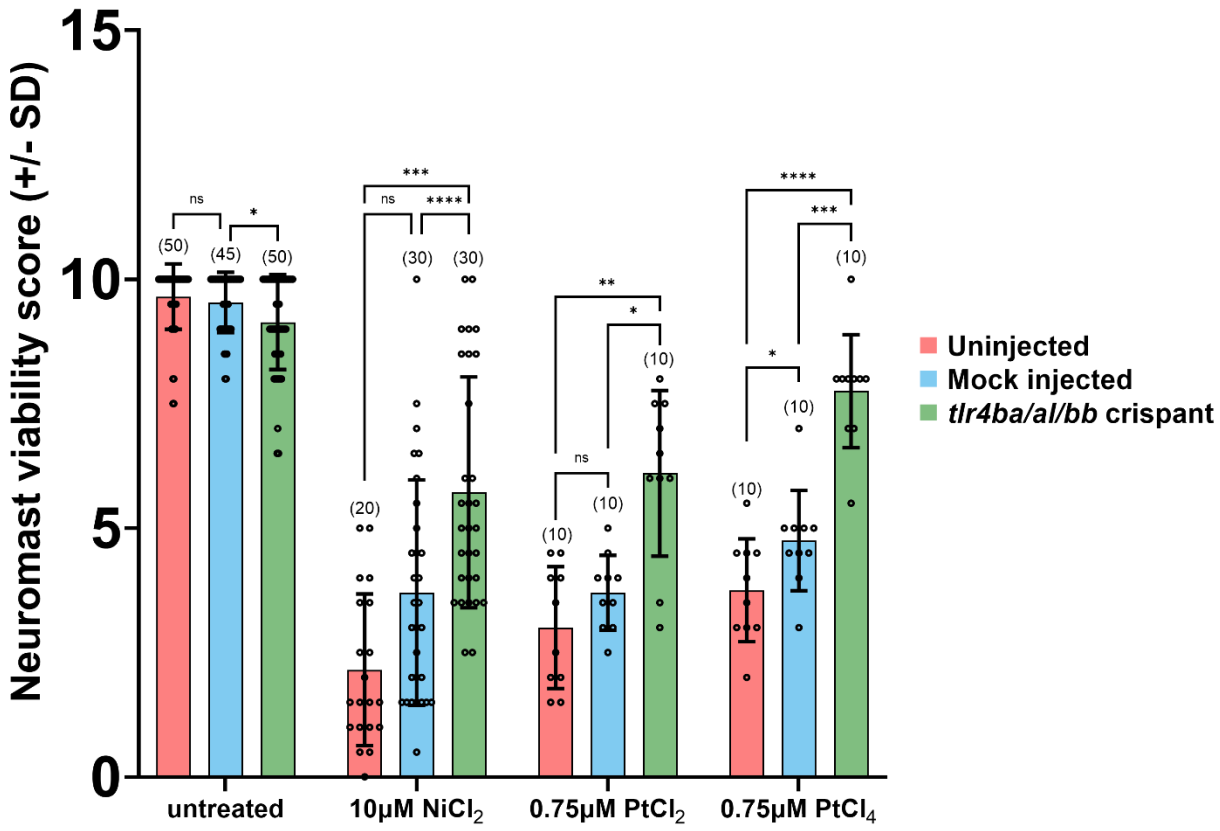


Figure 2. 14 *Tlr4 crispant* mutant larvae are less susceptible to Group 10 transition metal induced ototoxicity. Both uninjected and mock injected larvae show a significant decrease in neuromast hair cell survival after treatment with 10μM $NiCl_2$ and 0.75μM $PtCl_2$ and $PtCl_4$. The *Tlr4 crispant* larvae had their *tlr4* homologs mutated, protecting them from group 10 transition metal induced ototoxicity. Neuromasts showed a significant increase in viability compared to the mock and uninjected platinum treated groups. Notably, unlike *Tlr4 crispant*s treated with $PtCl_2$ and $PtCl_4$, crispant larvae treated with $NiCl_2$ did not show a significant increase in neuromast survival compared to mock and uninjected larvae. Larvae were mutated in their *tlr4* homologs using CRISPR-cas9 genome editing. Larva were injected at the one cell stage with either gRNA sequences targeting the three zebrafish tlr4 homologs or Cas9 protein alone (mock). Larvae were grown to 6 dpf when they were treated with cisplatin for 20 hours. Bracketed numbers above

each bar represents the total number of larvae scored in each group. ****$p<0.0001$, ns = not significant by one way ANOVA and Tukey's multiple comparison test.

Table 2. 1 CRISPR-cas9 gRNA sequences and PCR primer sequences.

Gene	gRNA sequence	PCR primer sequences
tlr4ba	5'-GGAGGUAUCUUACUAUGUUUGUUUUAGAGCUAU-3'	F: 5'-CCTGCATGGTCTTAATGTCAA-3'
		R: 5'-GCATTGATCATACAGCGAAAGA-3'
tlr4al	5'-UCCAAUCUCUCGAAGCACAAGUUUUAGAGCUAUGCU-3'	F: 5'-GACTCTTCCTCCATTCATGAGC-3'
		R: 5'-CAAACATCAGCCTTGTGACATT-3'
tlr4bb	5'-CAAACAACUUAUCCACGGUGGUUUUAGAGCUAU-3'	F: CCTTCATTTCGTTTAATGCACA-3'
		R: AACGAAACAAGTGCATTTCTGA- 3'

Chapter 3. Toll-like receptor 4 as a therapeutic target to reduce CIO in a zebrafish animal model.

Chapter 3 Preface:

Some of the research conducted in Chapter 3 is being prepared as a collaborative manuscript between the Bhavsar lab led by Dr. Amit P. Bhavsar at the University of Alberta, the Berman lab led by Dr. Jason Berman at the University of Ottawa, and the West lab led by Dr. Frederick G. West at the University of Alberta. At the time of writing, this manuscript in still being compiled, but this chapter was written by APDF with editing contributions from WTA. All in vivo work presented in this thesis was generated by APDF. Novel derivatives were generated by the West lab. The heterologous cell culture work was generated by Tracy Lee. Sakina Mithaiwala was instrumental in the generation of the YO-PRO1 assay and in helping establishing creation of the crispant mutant larva. Niall Pollock was fundamental in conceptualization and early instruction of fluorescent staining assays. Summary Table 3.1 represents in vitro cell culture data collected by Asna Latif and Ghazal Babolmorad. Christie Li generated gRNA sequences as well as PCR primers for crispant validation.

Abstract

Cisplatin (cis-diamminedichloroplatinum) is an effective anticancer drug used to treat a variety of solid tumours. Unfortunately, its use is limited by the onset of several side effects, particularly ototoxicity. Cisplatin induced ototoxicity (CIO) is irreversible, bilateral hearing loss that occurs in up to 90% of patients treated, depending mainly on cumulative dosage and age of treatment. Currently, mitigation strategies for the toxic side-effects of CIO are limited and only sodium thiosulfate has been approved by the FDA to date. CIO arises due to inflammation and generation of reactive oxygen species (ROS) within the closed system of the inner ear, leading to hair cell damage and apoptosis. The exact mechanism through which the inflammation and ROS generation are produced is still unknown. We propose TLR4 as a mechanistic link between cisplatin and generation of ROS within the inner ear, contributing to the release of proinflammatory cytokines and inner ear hair cell death. We use the zebrafish posterior lateral line as a model for inner ear hair cell death. Moreover, due to the unique binding mechanism of metals in comparison to LPS, we examine the ability of TLR4 inhibitors to selectively mitigate this side effect, while maintaining regular immune surveillance by TLR4. We establish cisplatin induces ototoxicity in a dose dependent manner, and that this effect can be mitigated by 2-fold using novel derivatives of the TLR4 inhibitor TAK-242. Moreover, we demonstrate that cisplatin can induce activation of zebrafish Tlr4 homologs by expressing them in vitro. We demonstrate zebrafish Tlr4 homologs are required for the generation of cisplatin-induced hair cell death and that our derivatives are specific for Tlr4 by using *Tlr4 crispant* mutant larvae. We also show that the hair cells protected by Tlr4 inhibition are functionally viable using a novel vibration startle response behavioural analysis. Altogether, our data provides further support of zebrafish as an

excellent model for studying CIO, screening drugs, and demonstrates TLR4 as a therapeutic target for combating cisplatin induced hearing loss.

3.1 Introduction

Cancer is one of the most prevalent and debilitating diseases worldwide with nearly 20 million people affected in 2020 and 10 million deaths ((IARC), 2020). Our ability to treat all cancer has improved considerably with the predicted 5-year survival rate for all cancers in 2017 being 64%, nearly 10% greater than in the late 20th century ((CCS), 2022). This puts more pressure on examining the effects of long-term cancer treatment. Cisplatin is one of the most broadly administered anticancer medication due to its effective and broad spectrum treatment for a variety of solid tumours (Aldossary, 2019). Cisplatin mainly exerts its apoptotic effects by targeting the DNA of cells, crosslinking base pairs which disrupts gene expression/replicaiton (Romani, 2022). This preferentially effects cells that divide quickly, such as cancer cells or hair follicles. However, cisplatin treatment is limited by several associated toxicities, particularly cisplatin-induced ototoxicity (CIO) (Waissbluth, Peleva, & Daniel, 2017). CIO is irreversible, bilateral, sensorineural hearing loss that is estimated to occur in 36% of adults and roughly 60% of pediatric patients (Chattaraj et al., 2023; Meijer et al., 2022). This toxicity often leads to a reduction or cessation of treatment, reducing patient survival and increasing their risk of relapse (Generotti et al., 2022; Rademaker-Lakhai et al., 2006). Not only does ototoxicity require an adjustment in treatment, but it has also been shown to have debilitating effects on the neurocognition of children and impairment to their psychosocial development (Moke et al., 2021).

Ototoxicity has gained a large amount of attention due to its irreversibility and life long determinantal effects. Ototoxicity occurs mainly due to the closed nature of the inner ear, where

cisplatin enters through the blood-labyrinth barrier and is unable to be flushed out leading to accumulation within cochlea (X. Wang et al., 2023). Once within the cochlea, cisplatin induces apoptosis through a wide variety of mechanisms, including the generation of reactive oxygen species (Clerici, DiMartino, & Prasad, 1995; Lee et al., 2004), inactivating antioxidant enzymes (Ravi, Somani, & Rybak, 1995), overexpression of ERK and NFκB (leading to increased levels of proinflammatory cytokines) (So et al., 2007), increased lipid peroxidation, and formation of DNA adducts. Previous research has indicated that an increase in proinflammatory cytokines such as TNF-alpha, IL-1β and IL-6 are directly related to cisplatin induced cell death (So et al., 2007). The inflammation following cisplatin treatment has been purposed as the triggering event for oxidative stress and reactive oxygen species (ROS) production (Gentilin et al., 2019). However, the exact mechanism responsible for the generation of these cytokines and inflammation is still to be determined.

Currently, only sodium thiosulfate (STS) is approved as a treatment for CIO by the Food and Drug Administration (FDA) ((FDA), 2022; Freyer et al., 2017). STS is known to bind the platinum molecule through its active thiol producing an inactive STS-cisplatin complex that is then excreted from the body (Wang et al., 2003). The STS-cisplatin complex may also help reduce ROS accumulation by mimicking antioxidant enzymes. Unfortunately, systemic administration of STS sequesters free cisplatin, therefore if it is used too early or during ongoing treatment, cisplatin efficacy is severely reduced (C.-H. Chen et al., 2021; Harned et al., 2008; Rybak et al., 2009; Sheth et al., 2017; Tang et al., 2021). Furthermore, the current practice of ototoxicity monitoring is poor and our ability to detect biomarkers for CIO before clinical symptoms is limited (Generotti et al., 2022). This leads to an urgent need of a preventative

otoprotectant therapy capable of mitigating this side effect, without interfering with cisplatin's antineoplastic effects.

A large range in therapeutic compounds have been examined for their otoprotective effects and have been summarized in depth (Guthrie & Spankovich, 2023). Most of these agents share the common characteristic of mimicking antioxidants, interacting directly with cisplatin to reduce ROS, or increase antioxidant activity. However, few of these otoprotective therapies act upstream to reduce inflammation and antioxidant production form the source. Recently, we proposed a potential pathway through which cisplatin induces an inflammatory response: by binding to the pattern recognition receptor (PRR) Toll-like receptor 4 (TLR4) (Babolmorad et al., 2021; Domingo et al., 2023). While TLR4 is canonically known to be a bacterial lipopolysaccharide (LPS) receptor, metal hypersensitivity reactions are also mediated by TLR4 in humans. These two ligand-types (LPS and transition metals) bind to the receptor in different ways. Binding of LPS to TLR4 is dependent on a co-receptor myeloid differentiation factor 2 (MD-2), while transition metals, such as Ni (II), have been reported to bind directly with the receptor via two histidine residues H456 and H458 (Peana et al., 2017; Rachmawati et al., 2013; Raghavan et al., 2012; Schmidt et al., 2010). Elements that fall within the same group on the periodic table are known to share similar chemical properties due to having the same number of valence electrons. Since cisplatin is a platinum based chemotherapeutic containing a coordinating PT (II) molecule, this provides further support for TLR4 acting as a receptor for this molecule. Furthermore, we have robust data showing TLR4 acts as a receptor for platinum (II) as well as cisplatin (Babolmorad et al., 2021; Domingo et al., 2023) (Chapter 2). The involvement of TLR4 in cisplatin induced ototoxicity reveals this PRR as a possible therapeutic target capable of mitigating this dose-limiting side effect. The disparity between the activation mechanism of

metals and LPS provides a basis for producing biased TLR4 inhibitors capable of preventing activation by metal ions, while maintaining the innate immune function of TLR4 as a bacterial sensor.

Due to the complexity and isolated location of the mechanosensory system of the inner ear, faithfully representing the biology in an accessible model is an ongoing challenge. We have chosen to use zebrafish as an animal model for this system and cisplatin-induced ototoxicity due to the presence of hair cells that are developmentally, morphologically, and functionally like hair cells within the human Organ of Corti (Coffin et al., 2010; Wertman et al., 2020). These hair cells, which are normally used for sensing water movement and vibration, akin to hearing, are found along the exterior of the fish within clusters called neuromasts (Thomas et al., 2015). Neuromasts are spread out in stereotypical positions over the head and body of the fish and are separated into two networks the anterior lateral line (ALL) and the posterior lateral line (pLL). The neuromasts of the lateral line are superficial and easily accessible to drugs and fluorescent dyes, making them an ideal screening system for in vivo ototoxicity. Other advantages of zebrafish are the quick generation time, large clutch size, small larval size, and natural transparency of the larvae allowing them to be used in high-throughput assays examining drug toxicity and protection of hair cells.

Zebrafish possess three tlr4-like gene paralogs (tlr4ba, tlr4bb, and tlr5al) and have recently been purposed as orthologous to human TLR4 (Babolmorad et al., 2021; Li et al., 2017; Loes et al., 2021). Recently, it was found that zebrafish tlr4ba can interact with an endogenous Md-2 homolog to activate NF-κB in response to LPS, indicating similarity to its human counter part (Loes et al., 2021). Moreover, their innate effector mechanisms are conserved with humans and chimeric studies in mice have shown that zebrafish Tlr4 (zebrafish Tlr4) homologs retain the

ability to induce NF-κB proinflammatory responses (Philip et al., 2017; Sullivan et al., 2009). Our previous data also shows zebrafish Tlr4 is required for CIO, once again supporting zebrafish as an excellent model for examining ototoxicity (Babolmorad et al., 2021).

Recently, TLR4 inhibitors have been identified as a plausible therapy for mitigating CIO by reducing inflammation, and therefore ROS generation (Babolmorad et al., 2021). TAK-242 (resatorvid) is an intracellular TLR4 inhibitor that works by binding to cysteine 747, preventing downstream signalling through the MyD88-depedent and -independent pathways when challenged by LPS (Matsunaga et al., 2011; Takashima et al., 2009). Cys747 is a highly conserved residue found between species, including zebrafish. Due to the distinct binding and activation mechanism of TLR4 by metals, we produced a variety of TAK-242 derivatives in the hopes that we could selectively inhibit metal ion binding, while allowing for canonical activation of TLR4 (Figure 3.1). We hypothesize that cisplatin can activate zebrafish Tlr4 and be selectively inhibited, allowing for regular immune surveillance and canonical signalling by LPS to remain. In this study, we examined the ability of these novel TLR4 inhibitors to reduce cell toxicity by cisplatin using both in vivo ototoxicity assays and an in vitro proinflammatory assay. By screening derivatives of TAK-242 we identified those best able to inhibit the activation of zebrafish Tlr4 and demonstrate reduced CIO in the zebrafish lateral line. Furthermore, we show that cisplatin induced deficits to vibration induced behavioural responses was protected by our novel derivatives. The findings of this study present novel inhibitors of TLR4 as a plausible therapy for CIO and demonstrate the ability of cisplatin to activate zebrafish Tlr4 homologs and induce cell death.

3.2 Methods

3.2.1 Zebrafish Husbandry and Ethics

All zebrafish were kept in the same facility and environment as those used for experiments in Chapter 2. For information on zebrafish husbandry, see Chapter 2, section 2.2.1.

3.2.2 Zebrafish breeding and care

Zebrafish breeding and maintenance was performed as described in Chapter 2, section 2.2.2. Briefly, Fish were bred at 28.5°C overnight, followed by embryo collection and storage in standard E3 media containing methylene blue. No more than 100 embryos were kept within a single petri dish for morphology experiments, while not more than 50 embryos for behavioural experiments.

3.2.3 Cisplatin and TAK-242 derivative treatments of larval zebrafish

Cisplatin and derivative treatment of larval zebrafish was in a similar manner to metal ion treatment in Chapter 2, section 2.2.3, with slight modifications. Briefly, 15 larvae were in each well of a six-well plate and pre-treated with for 1 hour with either, the vehicle (DMF; Fisher Scientific, cat. #D1331), TAK-242 (Cayman chemical, cat. #243984-11-4), or a derivative compound of choice (provided by Dr. Fredrick West, University of Alberta). These treatments were then washed using embryo media 3 times and then reintroduced at the same concentration along with either 0, 2.5, 5, 7.5, 10, or 15µM of cisplatin (TEVA, cat. #1848A001) for 20 hours at 28°C. These concentrations were chosen based off previous literature and pilot studies examining low dosages of cisplatin that induced partial, but not complete ablation of neuromast hair cells (Babolmorad et al., 2021; Wertman et al., 2020).

3.2.4 Neuromast quantification assay

The DASPEI quantification assay used here is the same as that used in Chapter 2, section 2.2.4. Briefly, five neuromasts along the posterior lateral line were chosen for scoring based on the scale previously described (Figure 3.2). Briefly, 6 dpf larvae were stained for 22 minutes using 0.01% DASPEI or for 30 minutes in 2µM Oxazole Yellow. Once larvae were anaesthetized, groups were blinded, and the posterior lateral line neuromasts were analyzed and imaged using a Leica M165 FC dissecting microscope equipped with a GFP-long pass fluorescent filter. When staining larva with YO-PRO-1, the individual hair cells within each neuromast were counted. The five neuromast hair cell counts were summed for each individual larva to produce a single data point.

3.2.5 Examining Neuromast Ultrastructure using SEM.

Zebrafish larvae were grown to 6-dpf and treated with cisplatin and TAK derivatives as described above. The larva was washed three times in E3 embryo media and transferred into labeled glass vials. They were then fully submerged within 2.5% glutaraldehyde and 2% PFA in 0.1M PBS overnight. In a fume hood, the fixative was decanted, and samples were washed with 0.1M PBS (pH 7.2) three times for 10 minutes each. Once washed, all samples were submerged in 1% osmium tetroxide for an hour to improve sample contrast. The samples were then washed again three times for 10 minutes in 0.1M PBS and dehydrated through an EtOH series. The series was as followed: 1x 30% ETOH for 15 minutes, 1x 50% EtOH for 15 minutes, 1x 70% EtOH for 15 minutes, 1x 90% EtOH for 15 minutes, 2x 100% EtOH for 10 minutes, and 1x 100% EtOH for 15 minutes. Once the final step of the ethanol dehydration was complete, it was decanted, and samples were dehydrated further in a series of HDMS (hexamethyldisilazane) washes. The HDMS series included a ratio of 75:25 ethanol:HDMS for 15 minutes, 50:50 for 15 minutes,

25:75 for 15 minutes, and 2X 100% HDMS for 15 minutes. The HDMS was poured off the samples, and the vials were left open in the fume hood overnight to air-dry. Dried samples were then mounted onto a scanning electron microscope (SEM) sample stub using double-sided sticky tape. They were then sputter coated with a gold/palladium mix and viewed under the SEM.

3.2.6 Guide RNA design and injections

Designing and injected the ribonuclease complexes was performed in the same manner as that previously described in Chapter 2, section 2.2.5. For information on crRNA sequences, see Table 2.1.

3.2.7 Confirming efficacy of tlr4 CRISPR via genotyping

Genotyping of Tlr4 crispant mutants was performed as previously described in Chapter 2, section 2.2.6. For information on the PCR primer sequences used, see Table 2.1.

3.2.8 HEK293T cell transfection and treatment

Transfection of HEK293T cells with zebrafish Tlr4ba and Tlr4bb homologs was performed as described in Chapter 2, section 2.2.7. 48 hours post transfection, media was aspirated, and the cells were treated with cisplatin (Pfizer, cat. #1848A001) or LPS (Invitrogen, cat. #L23351) diluted in fresh media.

3.2.9 HEK293T viability and IL-8 secretion

IL-8 secretion and cell viability was measured following cisplatin treatment in the same manner described in Chapter 2, section 2.2.9.

3.2.10 Examining neuromast rescue using vibration induced startle response

For behavioural experiments, the same method for cisplatin treatment was used as described in Chapter 2 section 2.2.3 with slight modification. 5 dpf larvae were pre-treated for

one hour in derivative 134 before being treated with either 0, 7.5µM, 15µM, or 50µM cisplatin for 20 hours. At 6 dpf, the wells of the six well plate were washed three times with methylene blue free E3 embryo media. Individual larvae from each group were then transferred into individual wells of a 24-well plate and topped with methylene blue free E3 embryo media.

After the larva were treated with cisplatin, the wells were washed three times with embryo media. Individual larvae from each group were then transferred into individual wells of a 24-well plate and topped with 3mL embryo media. A Zantiks codes for controlling the stimulus, video, and tracking of the larvae were written using notepad for windows. One 24-well plate containing a group of treated larvae was placed into the Zantiks MWP (multi-well plate) machine and the code was run. The code first shut off all lights within the machine to produce a dark environment. The larva's movement was tracked in both pixel number as well as millimeters for a 10-minute period allowing for larva to adapt to the environment. The software then recalibrated (referenced) the location of the larva and the first vibrational trial begun. A 30 second "pre-vibration" wait period tracked the larva's behaviour prior to the first vibration. The motor within the Zantiks machine then induced a one second vibration at a specified frequency and intensity. Frequencies included 1133.79Hz, 820Hz, 566.9Hz, 377.93Hz, 283.45Hz, 188.96Hz, 141.72Hz, 113.38Hz, 75Hz, 50Hz, and 25Hz. Intensities varied based on step size within the stepper motor producing the vibrations. From the lowest to the highest intensity, steps were as follows: an eighth of a step within the motor (I1), a quarter step (I2), a half step (I3), and a full step (I4). After the vibration, the larva was tracked during a 30 second "post-vibration" wait period to finish the first trial. Immediately following the first trial, two more identical vibration trials at the same frequency and intensity were administered.

Vibration frequency was programed based off the delay between motor steps within the zantiks box. The motor was coded to undergo 10 “steps” during one full turn. The delay time between each step was coded specifically to produce a range of vibrational frequencies. For example, if I wished to produce a frequency of 25Hz, the delay time was set to 0.04s between each step.

3.2.11 Statistical analysis

If using DASPEI, five individual neuromast scores were recorded on a scale of 0-2. When using YO-PRO1, hair cell counts for each of the five neuromast were recorded. Each DASPEI neuromast score was added together for each fish (ex. 2 + .5 + 1 + 1.5 + 0 = 5). On the other hand, YO-PRO1 hair cell counts from each neuromast was added together (ex. 9 + 12 + 8 + 10 + 10 = 49). The neuromast scores and hair cell counts from each fish was plotted as a single data point. The mean neuromast viability scores and hair cell count from each group were analyzed using one-way ANOVA with Tukey’s multiple comparisons test. All graphs and statistical tests were performed using GraphPad Prism 9. HEK293T normalized IL-8 secretion values were analyzed using an RM two-way ANOVA and Tukey’s multiple comparisons test.

For behavioural result analysis, all raw data was copied to excel. Startle distance in mm/s was binned into 30 second means for the initial 10-minute adaptation period and each pre- and post-vibrational period. For each larva, the startle distance (mm) for each of the three vibrational trials was adjusted by subtracting the average baseline distance moved in the previous 30 seconds from the peak vibrational startle distance. Therefore, there were 3 adjusted distances for each fish. These three adjusted distances were averaged for each fish and used as a single data point that was input into Prism GraphPad 9 where any outliers were removed using the ROUT

method at a threshold of 1. The cleaned data removing outliers was then analyzed using one-way ANOVA with Tukey's multiple comparisons test.

3.3 Results

3.3.1 Cisplatin is toxic to zebrafish neuromast hair cells in a dose-dependent manner.

The zebrafish posterior lateral line neuromasts contain hair cell bundles that are functionally and structurally like mammalian hair cells, allowing for a in vivo model of ototoxicity. To establish a working concentration of cisplatin in larval zebrafish, a dose response of neuromast hair cell health to cisplatin was made in the same manner as metals in chapter 2, except for the additional usage of YO-PRO1 (Section 2.3.1). Wildtype larvae were exposed overnight to either 0, 2.5, 5, 7.5, 10 and 15µM cisplatin dissolved in water and neuromast hair cell number and viability were examined. The no treatment group acted as a negative control to examine healthy neuromast fluorescence. Both neuromast viability and hair cell number decreased in a dose-dependent manner as cisplatin concentration increased (Figure 3.3A&B). At 7.5µM cisplatin, neuromast viability decreased 5.9-fold compared to the untreated group (Figure 3.3A). This is a similar outcome to what was observed in chapter two, and the same concentration chosen for nickel and cobalt (Chapter 2). When taking the mean hair cell count from the 5 neuromasts, hair cell number decreased from 40 in the untreated group to 8.3 in larvae treated with 7.5µM cisplatin (Figure 3.3B). At 15µM cisplatin, neuromast fluorescence was completely lost when staining with DASPEI, but when staining with YO-PRO1, the number of hair cells remained relatively unchanged after treatment with 7.5µM cisplatin. An untreated larva has healthy, bright fluorescent neuromasts which would be assigned a score of 2 for each of the five neuromasts chosen, but as cisplatin induces hair cell death, the fluorescence becomes fainter resulting in a lower score (Figure 3.3C&D). From these results, 7. 5µM cisplatin was chosen as

the concentration for future experiments due to its significant, but not complete ablation of neuromast hair cells.

Previous work has shown that there is a positive correlation with cisplatin concentration and damage to the functional structures of hair cells, such as the stereocilia (Comis et al., 1986). To examine the effects of cisplatin on the microstructure of the neuromasts, larvae were treated with cisplatin at 7.5, 15 and 50μM concentrations and processed for SEM imaging. Neuromasts contain organized bundles of kinocilia, which are lined at the base with rows of stereocilia organized in a staircase-like structure. The hair cells develop in pairs oriented 180 degrees to one another in an anterior and posterior manner in order to efficiently detect water flow from the front and back of the fish (Kindt, Finch, & Nicolson, 2012). Proper functioning of neuromasts, including sensing water flow, requires a combination of functional kinocilia and stereocilia. Larva treated with cisplatin showed increased disorganization and reduced structure of their P1 neuromast cilia in a dose dependent manner (Figure 3.3E-H). At 50μM cisplatin kinocilia showed reduced length and fusion of individual cilia with complete ablation of the stereocilia lining the base of the kinocilia (Figure 3.3H). Together, this data shows cisplatin induces neuromast hair cell death and destructive disorganization in neuromast cilia structure in a dose dependent manner.

3.3.2 TAK-242 derivative 134 mitigates cisplatin induced hair cell death in zebrafish larvae

To examine the involvement of TLR4 in cisplatin-induced ototoxicity, we began by testing the effects of the well-known intracellular TLR4 inhibitor TAK-242 on zebrafish. TAK-242 inhibits the intracellular signalling of TLR4 and subsequent release of proinflammatory cytokines and interferons, which would give insight into the effect of Tlr4 inhibition on cisplatin induced hair cell death in zebrafish. To determine which vehicle was best for the inhibitors, we

examined DMF, DMSO, and ethanol with and without cisplatin. Similar to Uribe et al., we found that DMSO was toxic to zebrafish in combination with cisplatin, while ethanol and DMF induced no change in neuromast viability (Figure 3.4A)(2013). To prevent any abnormal behaviour or muscle damage, we selected DMF as a vehicle for TAK-242 and the derivatives for future experiments (Bailey et al., 2015). Larvae were pre-treated with the TLR4 inhibitor TAK-242 followed by co-treatment with cisplatin. Interestingly, TAK-242 alone had no observable effect on neuromast hair cells, however when larvae were co-treated with TAK-242 and cisplatin, ototoxicity increased (Figure 3.4B).

On the other hand, the TAK-242 derivative 134 reduced CIO in 7dpf larvae in comparison to fish treated with cisplatin alone (Figure 3.4C&D). DASPEI stained larvae co-treated with 5μM 134 and cisplatin increased neuromast viability 2-fold compared to cisplatin alone, while 10μM 134 had no significant effect. However, higher concentrations of 134 were protective when examining neuromast hair cell survival. To help ensure these results were both reliable and valid, we examined hair cell damage using YO-PRO1 as a less subjective measure of neuromast health. Larvae stained with YO-PRO1 showed a 1.5-fold increase in neuromast hair cell survival when treated with 10μM 134 but did not show a significant increase when co-treated with 5μM 134. Larvae treated with both 5μM and 10μM 134 alone showed no observable change in phenotype or development (data not shown) and 5μM concentrations of 134 alone had no significant decrease in neuromast viability or hair cell count (Figure 3.4C&D). Notably, at higher concentrations of 134 (10μM), there is a slight decrease in neuromast viability and hair count. Together, this data indicated cisplatin displayed increased toxicity in combination with TAK-242 in zebrafish larvae, but its derivative, 134, eliminated this toxicity and displayed protection against the ototoxic effects of cisplatin.

3.3.3 Select TAK-242 derivatives are protective against cisplatin induced hair cell death in vivo, but not in vitro.

A total of fourteen TAK-242 derivatives were screened here. Each was produced by the collaborating laboratory led by Dr. Fred West. At the time of writing, the structures of these derivatives, remain proprietary. Each derivative was assessed for their selective ability to inhibit CIO. 7dpf larvae co-treated with 5µM derivative 136 and 7.5µM cisplatin increased neuromast viability 1.8-fold, but neuromast viability was not affected at higher concentrations of 136 (Figure 3.5A). Initial testing with 5µM and 10µM 136 alone showed a lack of toxicity to both the neuromasts and in general to the zebrafish, but later experiments showed a significant increase in toxicity (data not shown). This increased toxicity was reflected in a 1.5-fold decreased in neuromast viability at 5µM 136 alone and death of all larvae at 10µM 136 (Figure 3.5A). Interestingly, co-treatment of cisplatin with 136 decreased the toxicity to larvae. Co-treatment with 10µM 136 and 7.5µM cisplatin decreased larvae mortality allowing for 17 larvae to survive unlike larvae treated with 10µM 136 alone (Figure 3.5A).

Larvae treated with TAK-242 derivative 154 (10µM), 156 (5µM), 166 (5µM) showed a 1.6-, 2-, and 1.7-fold increase in neuromast viability respectively, when co-treated with 7.5µM cisplatin in comparison to cisplatin treatment alone (Figure 3.5B-D). None of these compounds were toxic to the larvae and showed no decrease in neuromast viability when 5µM or 10µM concentrations were applied on their own. Although 10µM 166 didn't show toxicity when treated alone, co-treatment with 7.5µM cisplatin resulted in the death of 16 of 20 larvae tested (Figure 3.5D). However, the four larvae that survived had significantly reduced CIO and increased neuromast viability. On the other hand, 10µM, but not 5µM 168 showed significant toxicity to 7dpf larvae resulting in death of all larvae treated at this concentration (data not shown).

However, lower concentrations of compound 168 (5µM) increased neuromast viability 3.4-fold when co-treated with cisplatin and showed low toxicity to zebrafish larvae (Figure 3.5E).

Four of the 14 TAK-242 derivatives (134, 136 166, and 168) were protective against CIO both in vitro and in vivo (Table 3.1). From our collaborator's investigations it was found that two of these four derivatives (134 and 136) were selective for protecting hair cells against CIO, while allowing for signalling to occur through the LPS-TLR4 complex in vitro (Table 3.1). Unfortunately, 136 showed toxicity to zebrafish larva in later trials, prompting us to select derivative 134 as the most promising derivative for selectively inhibit CIO in future experiments.

3.3.4 Some TAK-242 derivatives lack otoprotective effects and can be toxic to larval zebrafish

Five of the 14 TAK-242 derivatives tested on zebrafish showed no significant change in the level of CIO compared to larvae treated with cisplatin alone. Larvae treated with 150 (5µM), 158 (5µM), 162 (5µM and 10µM), 164 (5µM) and 170 (5µM) alone or in combination with 7.5µM cisplatin showed no significant change in neuromast viability (Figure 3.6A-E). However, treatment with a higher concentration of derivative 150 (10µM) alone significantly decreased neuromast viability and modestly increased CIO when co-treated with cisplatin (Figure 3.6A). Furthermore, higher concentrations of derivative 158 (10µM) and 164 (10µM) were toxic to zebrafish leading to death when applied alone or in combination with cisplatin (Figure 3.6B &D). Notably, 17/20 larvae were killed when co-treated with 10µM 158, but the three that survived showed slight decrease in neuromast viability.

Three of the 14 TAK-242 derivatives tested on zebrafish significantly increased the toxicity of cisplatin to zebrafish neuromast hair cells. Similar to their parent compound, derivative 120 (5µM) and 132 (10µM) showed no significant decrease in neuromast hair cell viability when applied alone, but significantly increased CIO when co-treated with cisplatin

(Figure 3.37&B). On the other hand, derivative 138 was toxic to zebrafish both alone and in combination with cisplatin (Figure 3.7C). Larvae treated with either 5µM or 10µM 138 alone died after treatment. However, when larvae were co-treated with cisplatin and 5µM 138, they all survived, but showed decreased neuromast viability. These results suggested some sort of protective ability of cisplatin against the more toxic TAK-242 derivatives.

3.3.5 Tlr4 homologs are required for both CIO and protection by derivative 134

To examine the contribution of Tlr4 activation to CIO in zebrafish larval hair cells, zebrafish Tlr4 homologs were mutated using CRISPR-cas9. Wildtype embryos were injected with three sgRNAs at the one cell stage targeting a sequence near the middle of the three Tlr4 genes, tlr4ba, tlr4al, and tlr4bb (Figure 2.10). We will refer to these mutant larvae as Tlr4 crispant larvae from this point forward. Uninjected, mock injected, and Tlr4 crispant larvae were grown to 5dpf and examined for morphological and developmental changes but showed no abnormalities (Figure 3.8). Furthermore, uninjected and injected larvae that were left untreated showed no change in neuromast viability. After being treated with 7.5µM cisplatin, Tlr4 crispants were protected against CIO in comparison to mock and uninjected larvae (Figure 3.9A&B). Following cisplatin treatment, neuromast viability increased 1.6-fold and mean neuromast hair cell count increased 1.7-fold in Tlr4 crispant larvae compared to mock injected larvae. This data demonstrates the requirement for zebrafish Tlr4 in CIO and provides further evidence for its use as a therapeutic target.

The way 134 was reducing cisplatin toxicity to neuromast hair cells was unknown. There is a possibility 134 was working in through a different mechanism than we predicted, for example, binding to another TLR, acting as an antioxidant, or complexing with cisplatin. To determine the specificity of 134 for TLR4, we utilized Tlr4 crispant larvae co-treated with both

134 and cisplatin. Uninjected, mock injected and Tlr4 crispant larvae were pre-treated with 5µM derivative 134 for one hour, followed by co-treatment with 7.5µM cisplatin for 20 hours. Mock injected larvae treated with 7.5µM cisplatin alone displayed a 2.7-fold decreased in neuromast viability compared to its untreated control (Figure 3.10A&B). This decrease was recovered by ~1.5-fold in both mock injected larvae treated with derivative 134 and in Tlr4 crispant larvae, showing the effects of derivative 134 are similar to mutating zebrafish *tlr4* homologs (Figure 3.7A, C, & D). Moreover, neuromasts had no additional protection when co-treating Tlr4 crispant larvae with 7.5µM cisplatin and 5µM 134 (Figure 3.10A & E). Thus, *TLR4* homologs are required for the action of 134 in protecting against CIO. Moreover, this demonstrates that 134 is specifically protecting CIO via *TLR4* homologs. Altogether, this data shows that protection from CIO by TAK-242 derivatives was mediated through zebrafish Tlr4 homologs and that zebrafish *tlr4* homologs are directly involved in development of cisplatin induced neuromast hair cell death.

3.3.6 Zebrafish Tlr4 homologs transfected into HEK293T cells respond to cisplatin, but not to LPS

Zebrafish Tlr4ba, Tlr4al, and Tlr4bb homologs share 37.29, 36.63, and 36.56 % identity respectively to human TLR4 at the amino acid level, and their downstream signalling pathways are shown to be highly conserved with mammalian TLR4 (Figure 2.3) (Campos-Sánchez & Esteban, 2021; Li et al., 2017; Sepulcre et al., 2009). Activation of zebrafish Tlr4 leads to expression of proinflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α by the zebrafish MyD88 dependent and independent pathways (Campos-Sánchez & Esteban, 2021; G.-p. Liu et al., 2018; Ren, Wang, & Hu, 2018; Zhang, Ren, & Chen, 2023). Therefore, to assess the ability of cisplatin to mediate the activation of the zebrafish Tlr4ba (zfTlr4ba) and zebrafish Tlr4bb

(zfTlr4bb), they were transfected individually on a recombinant plasmid into HEK293T cells and expressed using the polyomavirus simian virus 40 (SV40) promoter (Figure 2.8). IL-8 secretion values were normalized to baseline secretion levels and cells transfected with the empty vector alone acted as a negative control. Cells transfected with human TLR4 (hTLR4) are activated by both LPS (10 ng/mL) and cisplatin (25μM) without the need for MD-2. Cells transfected with hTLR4 alone increased IL-8 secretion 1.9-fold when challenged with 10ng/mL of LPS and 4.2-fold when challenged with 25μM cisplatin (Figure 3.11C). No significant increase in IL-8 secretion was observed when co-transfecting cells with hTLR4 and mMD-2 or hMD-2. In cells treated with LPS, co-transfecting hTLR4 with hMD-2 increased IL-8 release 3.1-fold compared to cells transfected with the empty vector alone, but co-transfecting hTLR4 with mMD-2 had no effect on IL-8 secretion. Co-transfection of hTL4 with hMD-2 allowed for a greater increase in IL-8 secretion when challenged with LPS compared to cells transfected with hTLR4 alone.

Using human cells as a comparison, cells transfected with zfTlr4ba are activated by cisplatin, but not LPS and its activation is dependent on the presence of an MD-2 homolog (Figure 3.11A). HEK293T cells co-transfected with zebrafish Tlr4ba and mouse MD-2 (mMD2) followed by treatment with cisplatin show a 1.7-fold increase in IL-8 secretion compared to cells transfected with the empty vector alone, while those co-transfected with human MD-2 (hMD2) show a 2.2-fold increase (Figure 3.11A).

Likewise, Tlr4bb activation is mediated by cisplatin and not LPS, however, its activation is independent of the presence of an MD-2 homolog (Figure 3.11B). HEK293T cells co-transfected with Tlr4bb and mMD-2 or hMD-2 and treated with cisplatin show a similar increase in IL-8 secretion to HEK293T cells transfected with Tlr4bb alone. Compared to cells transfected with only the empty vector, secretion of IL-8 increased 1.6-, 2.1-, and 1.9-fold in HEK293T cells

transfected with Tlr4bb alone, Tlr4bb + mMD2, and Tlr4bb + hMD2 respectively. No increase in IL-8 secretion was detected after LPS treatment in cells transfected with zebrafish Tlr4bb. Together, this data demonstrates the ability of cisplatin, but not LPS to activate zebrafish Tlr4 homologs in vitro, leading to an increase in the release of proinflammatory cytokine IL-8.

Overall, this data uses a heterologous expression system to demonstrate that zebrafish TLR4 homologs can mediate responses to cisplatin, in a manner that is similar to human TLR4.

3.3.7 Protection by 134 is functional following cisplatin insult

To determine if the hair cells rescued by derivative 134 from CIO are functional, we examined a behaviour mediated by the lateral line. The startle response is an escape behaviour dependent on external stimuli and mediated through the stimulation of neuromast hair cells (Han et al., 2020; Wang et al., 2017). The startle response distance of 5dpf wildtype larvae was recorded at different vibrational intensities and frequencies to examine if this behaviour is dependent on the intensity of the stimulus and could reliably measure neuromast function. At all frequencies tested, increasing the vibration intensity increased the mean startle response distance (Figure 3.12A). Larvae anaesthetized in MS-222 showed no response to vibration, as expected (Fig 3.12C). Cisplatin was then applied to 5dpf larvae at various doses to ablate hair cells and examine the effects on the startle response. For all frequencies, an inverse relationship between startle response distance and cisplatin concentration was observed. As the cisplatin concentration increased, the mean startle response distance decreased (Figure 3.12B). The highest average startle response distance was observed when applying stimuli in the lowest frequency range (25Hz-75Hz), while the middle frequency range (115Hz-380Hz) had the lowest average startle response distance. The average startle response distance in stimuli applied at the highest frequency range (570Hz-1135Hz) was intermediate to the low and middle frequency ranges.

After testing the 4 vibration intensities on cisplatin treated larvae, the highest intensity (I4) was selected for future experiments because it allowed for the greatest disparity between the distance swam by untreated larvae and cisplatin treated larvae.

We selected three vibration frequencies, representing the low (75Hz), middle (283Hz) and high (1135Hz) range, that showed a clear dose-dependent decrease in startle distance with cisplatin treatment (Figure 3.13A, C, E). We pretreated larvae for one hour with 5µM of the derivative 134 followed by co-treating with cisplatin and measured their responses to vibration stimuli. Derivative 134 protected the hair cells from CIO and recovered the startle response distance compared to larva treated with cisplatin alone (Figure 3.13B, D, F). Larvae treated with the vehicle (DMF), and larvae treated with TAK-242, were used as negative controls, while larvae anesthetized with 4% MS-222 acted as a positive control (Figure 3.12C). Two concentrations (7.5µM and 15µM) of cisplatin were chosen for testing neuromast hair cell function due to their ability to significantly reduce the startle response distance and to maintain the protective effects of 134 observed in previous morphological experiments. Derivative 134 had no significant affect on larval startle response distance on its own and did not reduce or increase startle response in comparison to untreated larvae (Figure 3.13B, D, F). With a vibration stimulus applied at 75Hz, the mean startle response distance modestly increases in larvae co-treated with 5µM 134 and 7.5µM cisplatin (Figure 3.12B). Similarly, larvae treated with 15µM cisplatin had no observable increase in the mean startle response distance at 75Hz. Stimulating larvae with a vibration in the middle frequency range (285Hz) showed the greatest recovery of startle response distance after co-treatment of 5µM 134 and 7.5µM cisplatin. The mean startle response distance at 285Hz increased from 2.9mm to 4.9mm when co-treated with 5µM 134, which is a full recovery of the startle response when compared to larvae treated with DMF alone

or with TAK-242 (Figure 3.13D). Larvae co-treated with 5μM 134 and 15μM cisplatin and stimulated at 285Hz also showed nearly a full recovery in startle response distance with 3.7mm traveled compared to 1.7mm in those treated with 15μM cisplatin alone. Although not significant, the mean startle response distance at 1135Hz modestly increased in larvae co-treated with 5μM 134 and 7.5μM cisplatin (Figure 3.13F). At 1135Hz, larvae treated with 15μM cisplatin showed no apparent increase in the startle response distance whether they were treated with 5μM 134 or not.

3.4 Discussion

Our previous work established that TLR4 is activated by platinum compounds, as well as cisplatin, and that this activation can be chemically inhibited to reduce CIO in vitro (Babolmorad et al., 2021). This study provides further evidence that cisplatin mediates the activation of Tlr4 homologs in zebrafish and demonstrates the potential of this receptor as a therapeutic target for reducing CIO in vivo. We showed that bath application of cisplatin induced neuromast hair cell death in a dose-dependent manner and that this effect required zebrafish Tlr4 homologs. Furthermore, cells transfected with zebrafish Tlr4 were activated in the presence of cisplatin, but not LPS. We also demonstrated the protective ability of novel TAK-242 derivatives in mitigating CIO within zebrafish larva, and that these effects were mediated through zebrafish Tlr4 homologs. Moreover, the protection from CIO provided by derivative 134 was shown to be functional, improving the ability of larva to detect external stimuli. Altogether, this data suggests that inhibition of zebrafish Tlr4 homologs protects larva from cisplatin induced hair cell death.

Currently, most substances examined for their protective ability against cisplatin-induced hair cell death aim to reduce oxidative stress (Domarecka et al., 2020). One of the major mechanisms believed to induce apoptosis within cochlear hair cells is an overproduction in ROS

(Huang et al., 2000; Lu et al., 2022). While multiple processes likely contribute to this increase, the signal responsible for initiating increased ROS generation is still poorly understood. Toll-like receptors such as TLR1, TLR2 and TLR4 have been shown to trigger ROS production upon stimulation with their respective agonists (Li & Chang, 2021; West et al., 2011). This led us to hypothesize the involvement of a PRR in the initiation of ROS generation, and later, Domingo et al. found that TLR4 can directly bind to cisplatin, leading to its activation (2023). Moreover, we have recently shown that TLR4 acts to mediate CIO, inducing activation of MyD88-dependent and independent pathways that lead to the generation of proinflammatory cytokines and ROS (Babolmorad et al., 2021). Our results support these findings, and further show that cisplatin induces CIO in vivo and that this is directly linked to the proper expression of Tlr4 (Figure 3.3; Figure 3.9). This opens the possibility of using TLR4 as a therapeutic target for the mitigation of cisplatin induced hair cell death.

Babolmorad et al. further validated their findings that TLR4 mediated CIO by demonstrating the protective ability of chemically inhibiting TLR4 with the small molecule TAK-242, reducing TLR4 activation and ROS generation in HEI-OC1 cells in the presence of cisplatin (2021). Unfortunately, the inhibition also reduced activation in the presence of TLR4's canonical ligand LPS, which would impede the ability of the immune system to be warned of present bacterial infections. TAK-242 selectively inhibits TLR4 signalling by covalently binding to the intracellular TIR domain via Cys747, preventing interaction with adaptor molecules TIRAP and TRAM (Figure 3.1) (Matsunaga et al., 2011). While the exact binding mechanism is poorly understood, the α, β-unsaturated carbonyl within TAK-242 makes it a good candidate to act as a Michael acceptor, binding the nucleophilic thiol of cysteine (Patra et al., 2018; Peri & Calabrese, 2014; Takashima et al., 2009). Previous studies have established that TLR4 binds to

metals such as nickel and cobalt, in a distinct mechanism from other canonical ligands like LPS and HMGB1 (Peana et al., 2017; Raghavan et al., 2012; Schmidt et al., 2010; Yang et al., 2015). We produced structural derivatives of TAK-242 to modify its interactions with the TIR domain of TLR4. These derivatives aim to assess if we can selectively inhibit metal binding of TLR4 with structural modification to TAK-242. Our in vitro cell culture experiments showed multiple derivatives were successful in preventing hair cell death from cisplatin, while conserving LPS activation (Table 3.1; data not shown). The mechanisms behind the selective nature of these novel derivatives are still unknown but will be a focus for future studies.

Although zebrafish are known to display low sensitivity to LPS and do not primarily respond to this PAMP through Tlr4 like mammals, they still express all the necessary components required to for metal activation of Tlr4 and share conserved structure and intracellular signalling cascades (Loes et al., 2021; Schmidt et al., 2010). Moreover, a previous study by Lee et al. showed that cisplatin treatment of larva induced an inflammatory response within lateral line neuromast hair cells (2022). Our results provide further insight to this finding using an in vitro cell culture assay, where we show that zebrafish Tlr4 homologs are activated in the presence of cisplatin, but lack of a response to LPS (Figure 3.11A & B). This demonstrates the potential of zebrafish Tlr4 homologs in revealing the efficacy of these new inhibitors to prevent metal mediated Tlr4 activation. Fourteen derivatives were tested for their inhibitory effects on cisplatin induced neuromast hair cell death, with only six of them being otoprotective, and only two showing biases in vitro towards inhibiting metal activation only (Table 3.1; Figure 3.4 & 3.5). We show that derivative 134 provides significant protection to neuromast hair cells and that this activation is principally mediated through Tlr4 because crispant mutants lacking proper expression of these homologs showed no additional protection from derivative 134

(Figure 3.4C & D; Figure3.10). While preliminary in vitro cell culture experiments begin to demonstrate the selective ability of the TAK-242 derivatives, this study does not show the selectivity of these derivatives in vivo and further study, potentially in mice, is needed to show this.

One of the major benefits of working with an animal model during drug discovery is the ability to observe off target effects that arise from the interactions between different cell types and tissues, while also examining the efficacy of the drug in producing the desired outcome. Five of the derivatives (150, 158, 162, 164, 170) failed to induce any significant changes in the levels of ototoxicity induced by cisplatin both in vivo and in vitro (Table 3.1). Four of five of these derivatives were specific for reducing LPS activation of TLR4 in vitro, which may explain why no effect was observed in zebrafish, since they are known to be insensitive to LPS (Sepulcre et al., 2009). On the other hand, ten of the derivatives either furthered ototoxicity or induced generalized toxicity leading to death of the larva. However, only one derivative (138) was also toxic to cells in vitro, which may indicate that the increase in toxicity was due to a process not found in cell culture, such as drug metabolism. Interestingly, some derivatives (136, 138, 158) showed reduced toxicity in larva when co-treated with cisplatin compared to when they were used alone (Figure 3.5; Figure 3.6). The reduced toxicity may be due to a direct interaction between the derivatives and cisplatin, or a change induced by cisplatin reduced the sensitivity of the larva to the derivatives. We do know that when our Tlr4 crispants were co-treated with 134 and cisplatin, there was no observable difference in protection compared to 134 alone (Figure 3.10). Therefore, the action of 134 must be principally mediated through Tlr4. What ever the case, cisplatin is known to bind to different proteins beyond TLR4 and take part in different

metabolic pathways (Choi et al., 2015; Messori & Merlino, 2016; Zhang & Hanigan, 2003), but the mechanism behind reduced derivative toxicity by cisplatin requires further study.

Examination of CIO using fluorescent vital dyes in the lateral line neuromasts demonstrates the protective ability of these derivatives by reducing structural damage. However, this does not provide insight into the functionality of the protected surviving neuromasts following cisplatin insult. Behavioural assays can also help determine whether anatomically preserved/rescued hair cells are truly functional, complimenting anatomic assays. Previous studies established that startle response is mediated through the lateral line and that ototoxic assault reduces the intensity of this response (Buck et al., 2012; Han et al., 2020). Our data shows that cisplatin induced hair cell death within the lateral line neuromasts significantly reduces larval zebrafish startle response distance in a dose dependent manner (Figure 3.12B; Figure 3.13A, C, E). While behaviour is a multifaceted process that results from a culmination of many different inputs, it is hard to directly contribute neuromast hair cell function to a decrease in movement. Our results show that the baseline movement of fish was unaffected by cisplatin, the vehicle (DMF), or the derivatives alone or in combination with one another (Figure 3.16). This suggests that these compounds had little effect on normal behaviour of larva. Furthermore, we show that protection of lateral line neuromast hair cells by derivative 134 was able to successfully recover startle response distance, while having no effect on startle distance when applied alone (Figure 13B, D, F). Some studies have shown that only a small number of functional neuromast hair cells are required for proper detection of stimuli in the lateral line (Buck et al., 2012; Q. Zhang et al., 2018). Therefore, two possibilities may help explain our results. First, it is possible that cisplatin insult only kills a subset of hair cells, but still induces significant damage in surviving hair cells, therefore preventing proper signalling by neuromasts,

and limiting the startle response. These living but damaged hair cells were protected by the derivatives, facilitating a recovery in the startle response distance. Second, cisplatin-induced hair cell death within neuromasts decreased their ability to detect of stimuli, reducing their startle response distance in a similar manner to a reduction in the stimuli intensity (Figure 3.12A). The protection provided by derivative 134 increased the total number of surviving hair cells, allowing for a full detection of the stimuli and a recovery in the response distance. Altogether, our results suggest that protecting hair cells from cisplatin by inhibiting Tlr4 decreases neuromast hair cell death and that the protected hair cells retain their function.

In summary, our data demonstrates that zebrafish Tlr4 can be used as a therapeutic target to selectively mitigate CIO. These findings provide support to our previous publication demonstrating that TLR4 contributes to CIO both in vitro and in vivo (Babolmorad et al., 2021). We also have also provided support to for unique interaction between zebrafish Tlr4 and cisplatin, which gives insight into the function of these currently orphaned receptors. One big question remaining is whether cisplatin efficacy is maintained with co-treatment of a derivative. Our collaborators in the Berman Lab are currently looking into this possibility and will be expanded upon in the next chapter. Moreover, these findings may also have implications for patients suffering from metal allergies like contact hypersensitivity, providing an avenue for the design of future therapeutics.

3.5 Figures and Tables

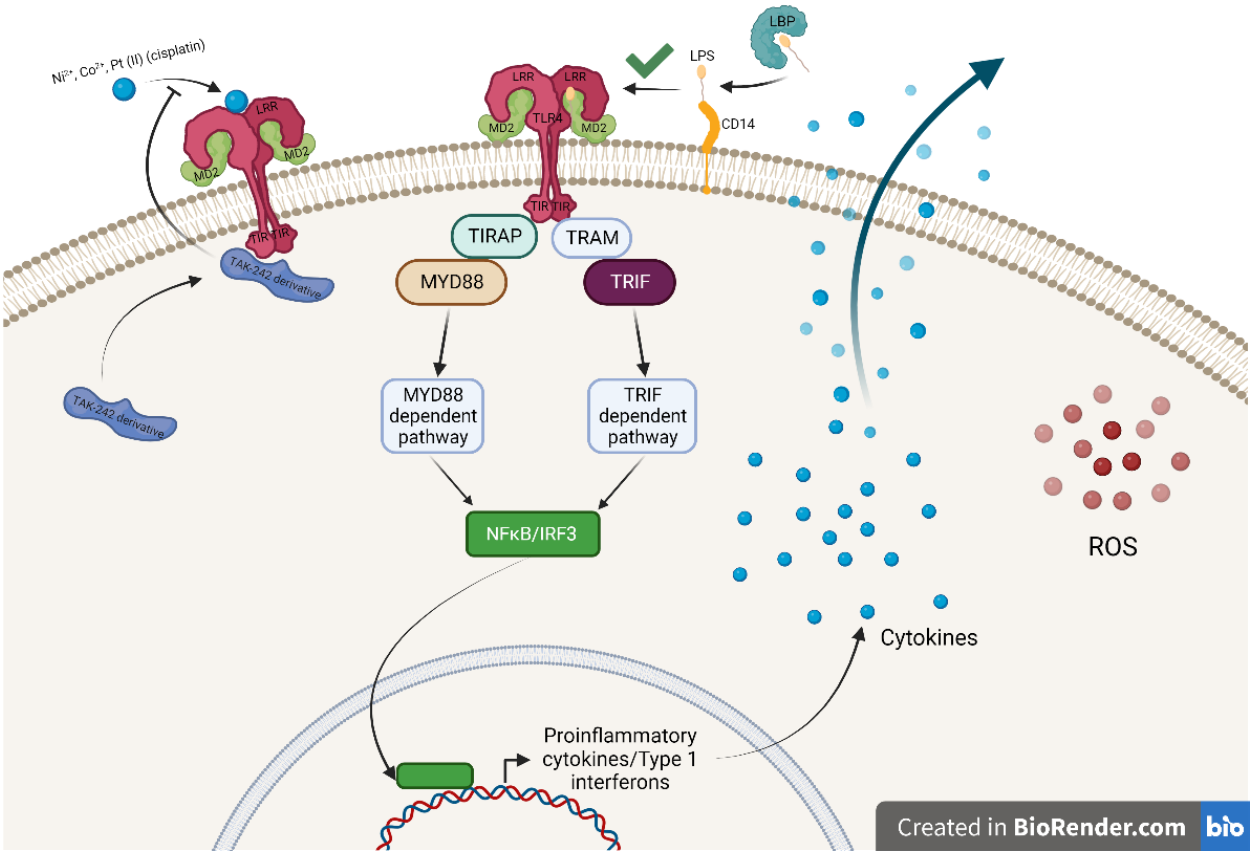


Figure 3. 1 Inhibition of Tlr4 signalling by synthetic TAK-242 derivatives. LPS uses co-receptor CD14 and MD-2 to bind, dimerize, and initiate signalling through TLR4. This stimulates both the MYD88 dependent and MYD88 independent pathways leading to activation of TFN release of proinflammatory cytokines and type 1 interferons. Group 10 metals do not require a co-receptor MD-2 to initiate binding to the ectodomain of TLR4. TAK-242 is a well-known small molecule inhibitor of the intracellular region of TLR4, capable of inhibiting LPS signalling through both MYD88 and TRIF-dependent pathways. 14 novel derivatives of TAK-242 were produced to inhibit metal ion mediated TLR4 signalling (left), while allowing for other

TLR4 agonists to continue to bind and activate this crucial immune receptor (right). Created with BioRender.

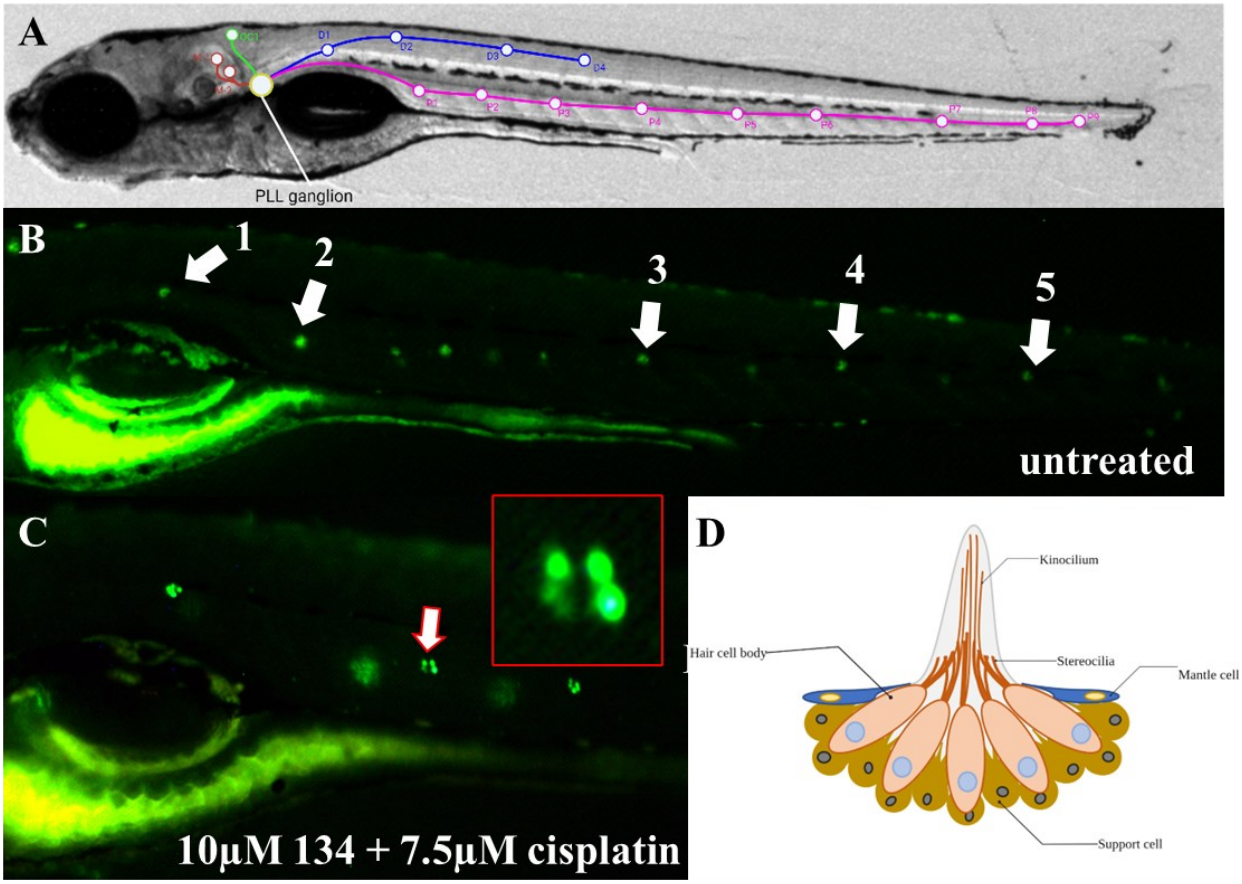


Figure 3. 2 Depiction of the zebrafish posterior lateral line and neuromasts in 7dpf zebrafish larvae. A) Location of posterior lateral line neuromasts drawn on a bright field image of a 7dpf larva. B) DASPEI stained untreated larva with visible neuromasts. White arrows point towards the location of the five stereotypically developed neuromasts consistently scored between experiments. C) YO-PRO1 stained larva co-treated with TAK-242 antagonist 134 and cisplatin showing the fluorescence of individual neuromast hair cells. The red arrow indicates a neuromast that would have been scored, along with a zoomed-in image of said neuromast in the top right corner. D) Cartoon diagram of a zebrafish neuromast illustrating the different cell types including the bundle of hair cells (peach), surrounding support cells (brown) and mantle cells

(blue). Stereocilia and kinocilia protrude out of the body, surrounded by a fluid filled cupula, and are used to detect water flow.

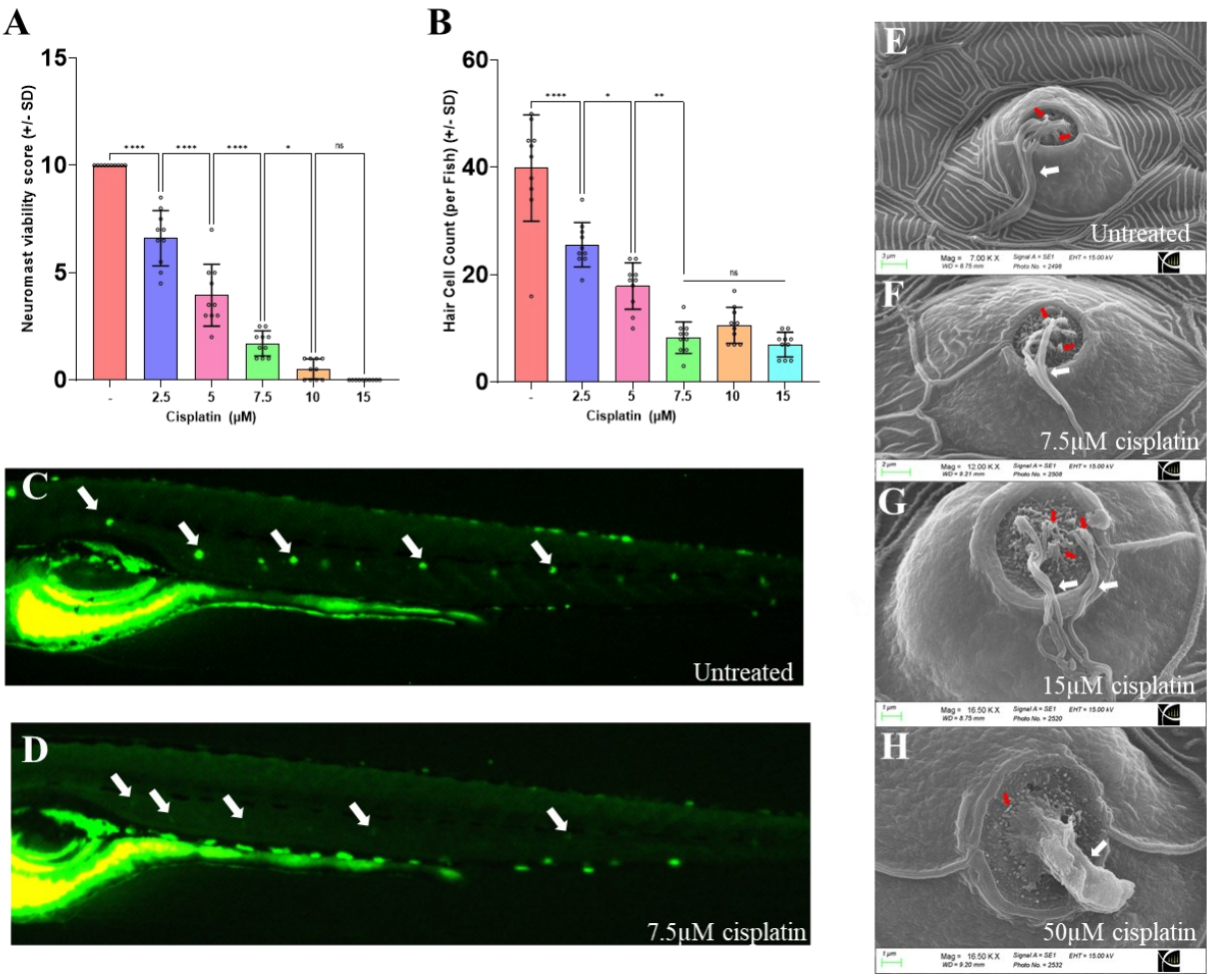


Figure 3. 3 Dose-dependent effect of cisplatin on larval zebrafish neuromast hair cell survival. 6dpf zebrafish were incubated for 20 hours in varying concentrations of cisplatin. A) Neuromast viability scored based on DASPEI fluorescence. Each data point (dots) represents the total neuromast viability score for a single larva, with a lower score representing more hair cell death. Increasing cisplatin dose decreases hair cell viability (fluorescence). B) Neuromast hair cell abundance detected with YO-PRO-1. Each data point represents the total hair cell count for a single larva. Hair cell survival decreases as cisplatin concentration increases. C-D) Fluorescent images of DASPEI stained larva with visible posterior lateral line neuromasts in an untreated (C) and cisplatin treated (D) larvae. E-H) SEM images reveal the microstructure of the P1 posterior

lateral line neuromasts after being treated with increasing cisplatin concentrations. White arrows show kinocilia slowly becoming more disorganized and structured as cisplatin concentration increases. Red arrows show the ablation of stereocilia with an increase in cisplatin. N = 10 larva for all treatment groups. Bars represent the mean neuromast viability score of all larvae. ****$p<0.0001$, ***$p<0.0002$, **$p<0.0021$, *$p<0.0332$, ns = not significant by one way ANOVA and Tukey's multiple comparison test.

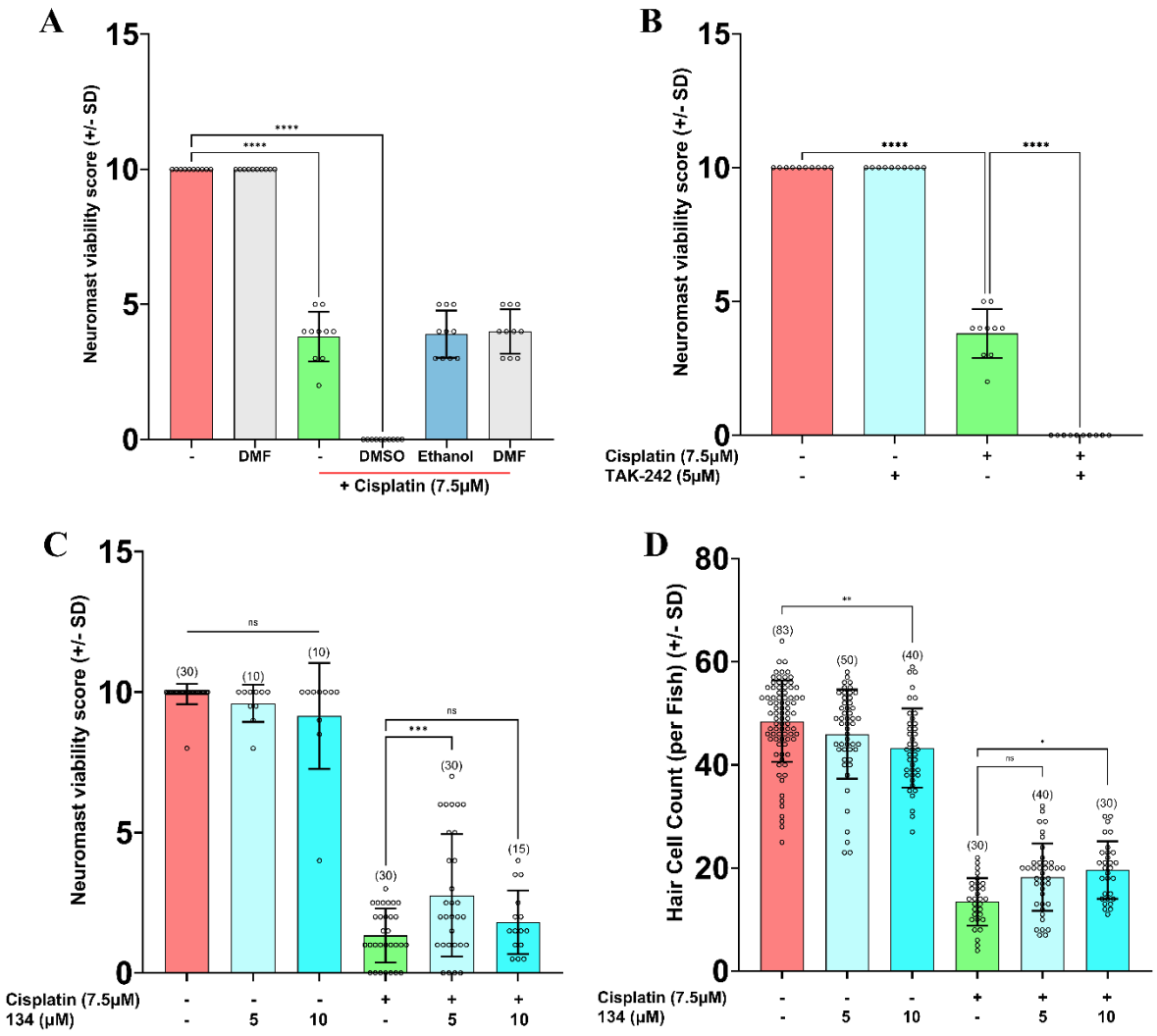


Figure 3. 4 TAK-242 derivative 134 provides protection from cisplatin-induced ototoxicity in larval zebrafish, but TAK-242 does not. DMSO exacerbates cisplatin ototoxicity when co-treated with cisplatin, while ethanol and DMF had no additional effect. 6-7dpf zebrafish were pre-treated for 1 hour in each vehicle, followed by a 20-hour co-treatment with cisplatin. A) Larvae stained with DASPEI exhibited low fluorescent scores following co-treatment with DMSO and cisplatin, but similar scores to cisplatin alone with a variety of other vehicles for TAK-242 and its derivatives. B) TLR4 antagonist TAK-242 alone does not influence neuromast hair cell viability, but larvae co-treated with TAK-242 and cisplatin results in an increase in

neuromast hair cell death. C-D) 6-7 dpf zebrafish larvae were pre-treated for one hour in either 5μM or 10μM 134 before co-treatment for 20 hours with cisplatin. Reduction of CIO in zebrafish larvae by the TAK-242 derivative 134 was apparent at 5μM when quantified via DASPEI staining (C), and 10μM when quantified via YO-PRO1 staining. Bracketed numbers above each bar represents the total number of larvae in each treatment group. ****$p<0.0001$, ***$p<0.0002$, **$p<0.0021$, *$p<0.0332$, ns = not significant by one way ANOVA and Tukey's multiple comparison test.

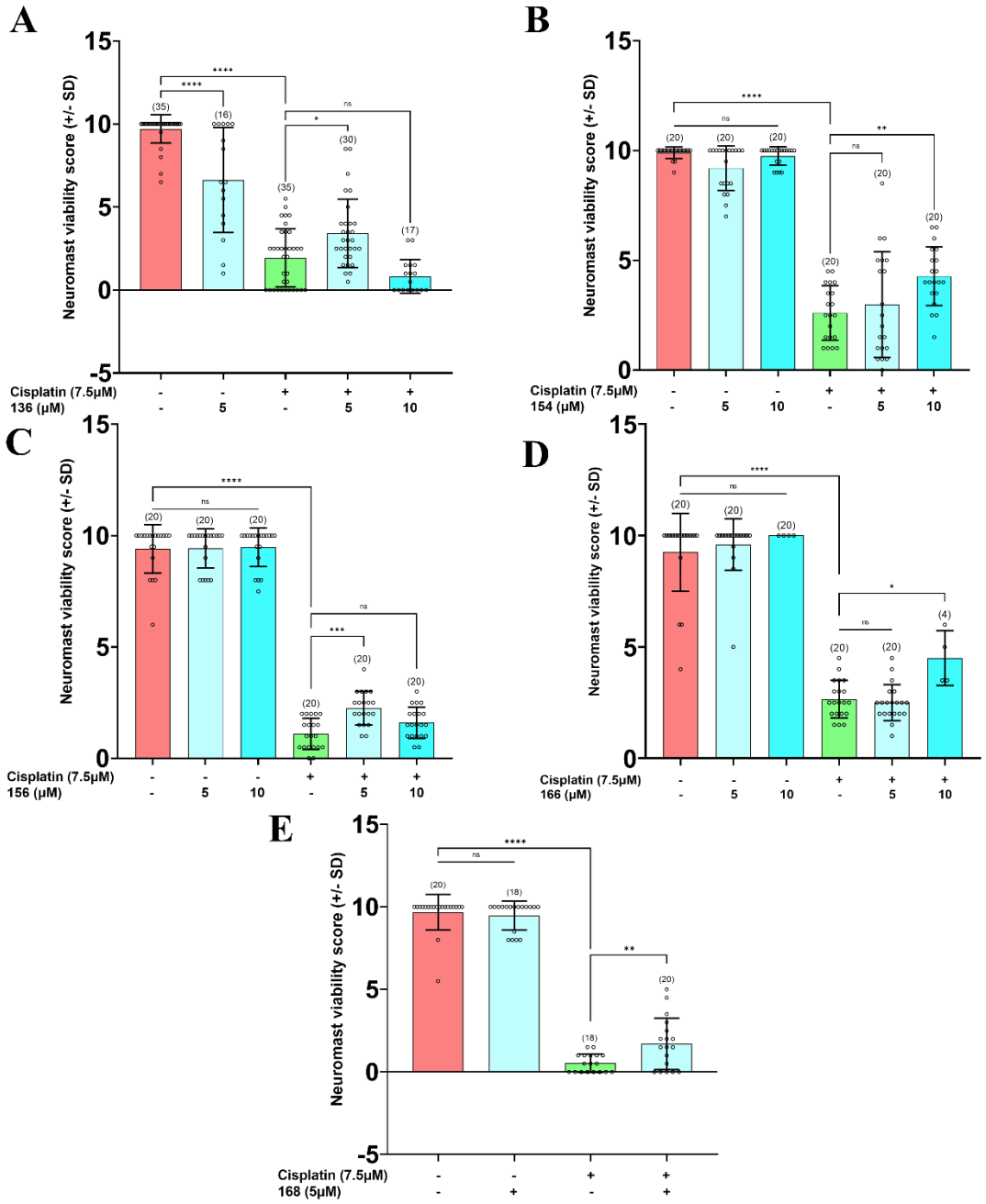


Figure 3. 5 Multiple TAK-242 derivatives are otoprotective against cisplatin in zebrafish larvae. 6-7 dpf zebrafish larvae were pre-treated for one hour in varying concentrations of 136 (A), 154 (B), 156 (C), 166 (D), and 168 (E) before co-treatment for 20 hours with cisplatin. A) Derivative 136 (5μM) shows a significant increase in neuromast hair cell viability when co-

treated with cisplatin, but also shows significant toxicity to neuromast hair cells when applied alone. Notably, all larvae died when applying 10μM of 136 alone, but when applied with cisplatin, 17 of 26 larvae survived. However, larvae co-treated with 10μM 136 and 7.5μM cisplatin showed a decrease in neuromast hair cell viability. B) Co-treatment with 10μM 154, but not 5μM 154 significantly increases neuromast hair cell viability. C) Co-treatment with 5μM 156 significantly increases neuromast hair cell viability in comparison to cisplatin treatment alone. Increasing the concentration to10μM 156 reduces the protective affect when applied with cisplatin. D) Co-treatment with 10μM 166, but not 5μM 166 significantly increases neuromast hair cell viability in comparison to cisplatin treatment alone. However, a significant number of larvae were killed during treatment with 10μM 166 and cisplatin. E) Co-treatment with 5μM 168 significantly increases neuromast hair cell viability in comparison to cisplatin treatment alone. All larvae were stained with DASPEI and scored using a fluorescent microscope. Bracketed numbers above each bar represents the total number of larvae scored in each group. ****$p<0.0001$, ***$p<0.0002$, **$p<0.0021$, *$p<0.0332$, ns = not significant by one way ANOVA and Tukey's multiple comparison test.

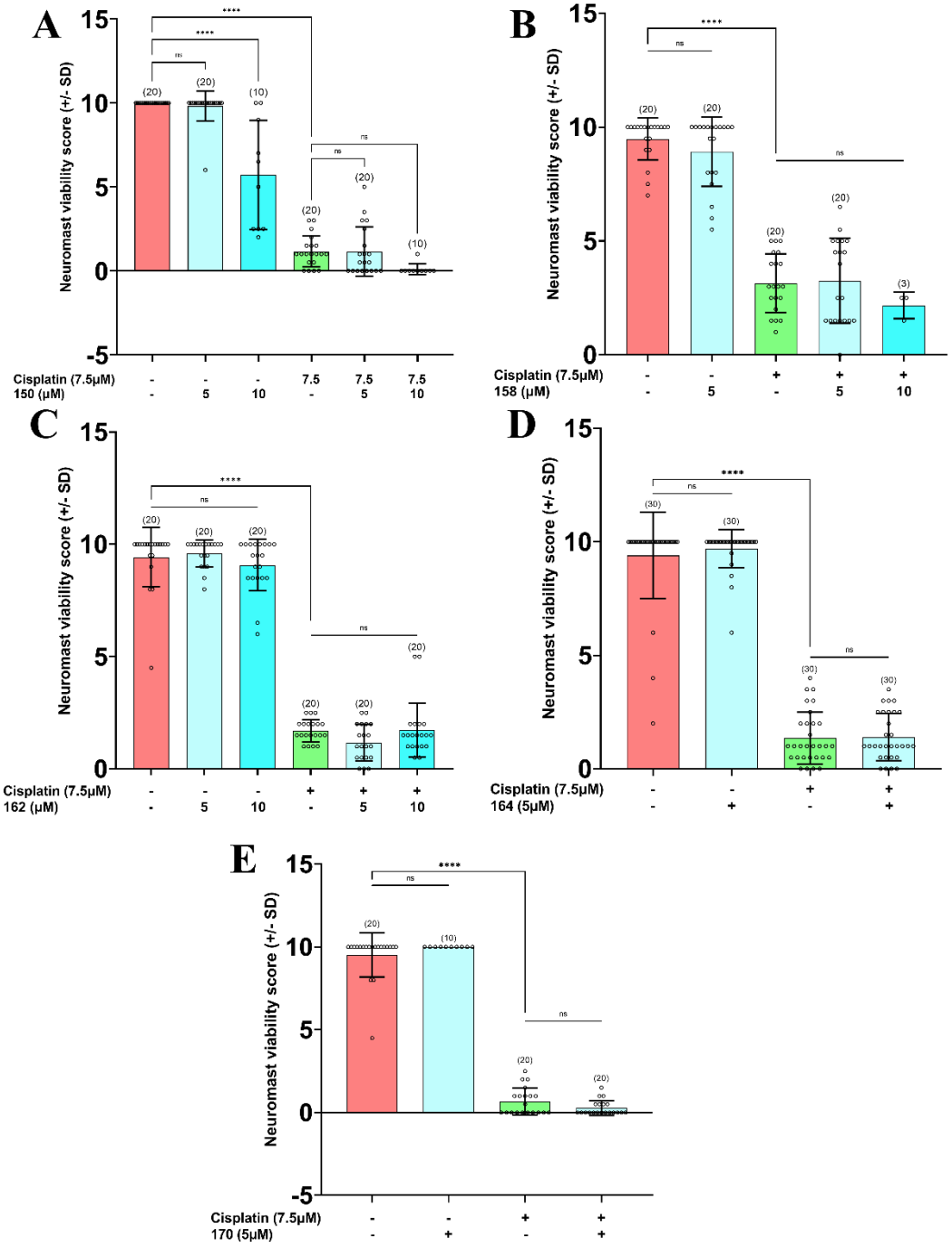


Figure 3. 6 Multiple TAK-242 derivatives have no effect on ototoxicity from cisplatin in zebrafish larvae. 6-7 dpf zebrafish larvae were pre-treated for one hour in varying concentrations of 150 (A), 158 (B), 162 (C), 164 (D), and 170 (E) before co-treatment for 20

hours with cisplatin. A) Derivative 150 at 5μM shows no significant affect to neuromast viability when applied alone and no increase in protection from CIO when co-treated with cisplatin. However, 10μM of 150 shows a significant decrease in neuromast viability when applied alone, as well as a decrease in neuromast viability when co-treated with cisplatin. B) Larvae treated with 158 show no significant increase in protection from CIO when co-treated with cisplatin at both 5μM and 10μM concentrations. Treatment with 5μM 158 alone had no significant affect on neuromast viability, but 10μM treatment alone resulted in death of all larvae. Derivatives 162 (C), 164 (D), and 170 (E) show no significant increase in protection from CIO when co-treated with cisplatin, but also show toxicity to neuromast hair cells when applied alone. All larvae were stained with DASPEI and scored using a fluorescent microscope. Bracketed numbers above each bar represents the total number of larvae scored in each group. ****$p<0.0001$, ***$p<0.0002$, **$p<0.0021$, *$p<0.0332$, ns = not significant by one way ANOVA and Tukey's multiple comparison test.

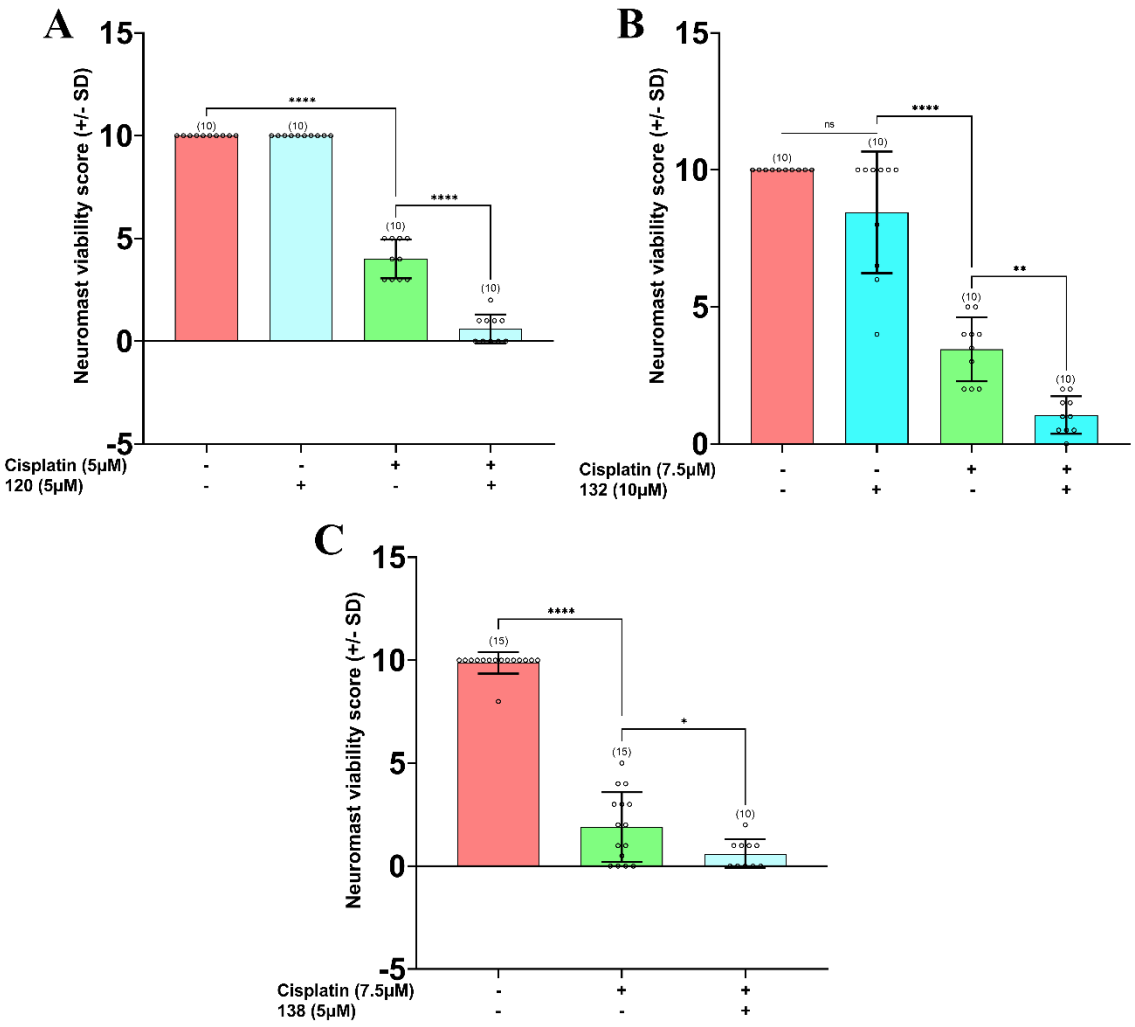


Figure 3. 7 Multiple TAK-242 derivatives increase the ototoxic effects of cisplatin in zebrafish larvae. 6-7 dpf zebrafish larvae were pre-treated for one hour in varying concentrations of 120 (A), 132 (B), and 138 (C) before co-treatment for 20 hours with cisplatin. Derivatives 120 (5μM) (A), 132 (10μM) (B), and 138 (5μM) (C) show a significant decrease in neuromast hair cell viability when co-treated with cisplatin. B) There was a modest decrease in neuromast viability when larva was treated with 132 alone. C) 138 (5μM) was found to be toxic to larvae not only when co-treated with cisplatin, but also when applied alone. All larvae died when applying 5μM of 138 alone, but when applied with cisplatin, all larvae survived. All larvae

were stained with DASPEI and scored using a fluorescent microscope. Bracketed numbers above each bar represents the total number of larvae scored in each group. The data in panel A and B were supplied by Niall M. Pollock. ****$p<0.0001$, ***$p<0.0002$, **$p<0.0021$, *$p<0.0332$, ns = not significant by one way ANOVA and Tukey's multiple comparison test.

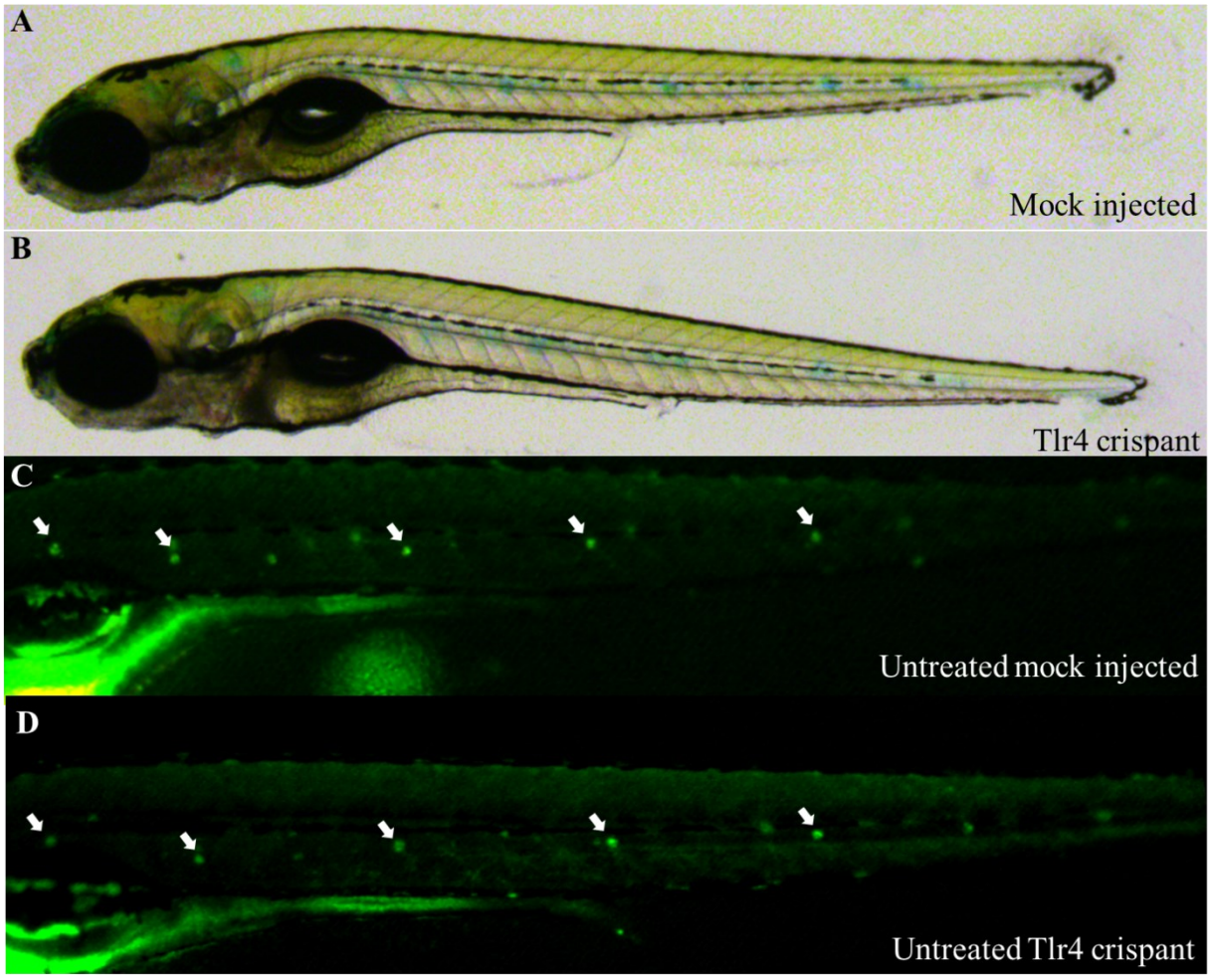


Figure 3. 8 Morphology of 7dpf Tlr4 crispant mutants. A-B) Comparison between the lateral view of a mock injected and Tlr4 crispant mutant larva show no notable differences in the morphology. C-D) The posterior lateral line of Tlr4 crispant mutants stained with DASPEI demonstrates normal development and deposition of neuromasts when compared to a mock injected larva. White arrows point towards healthy neuromasts scored during morphology experiments.

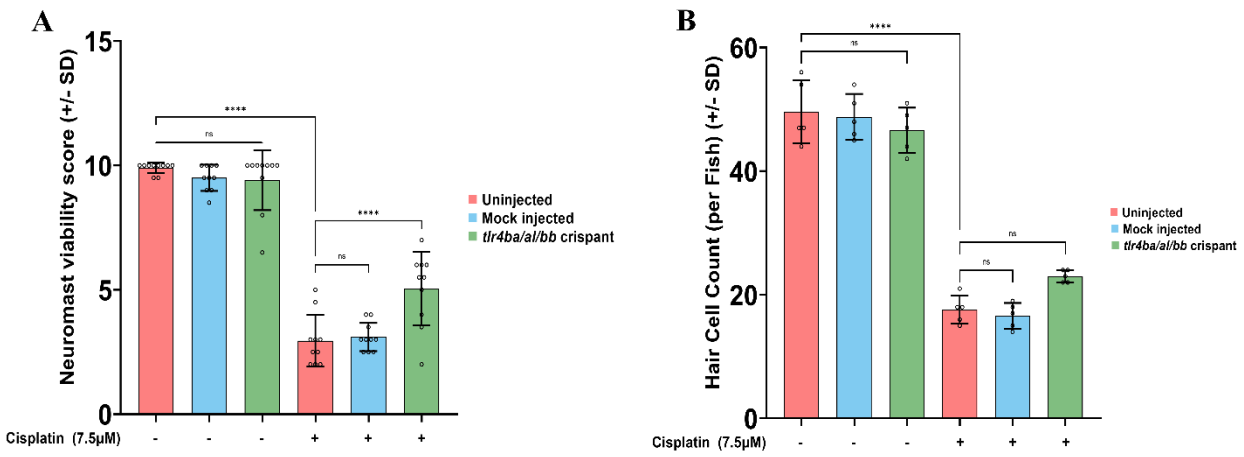
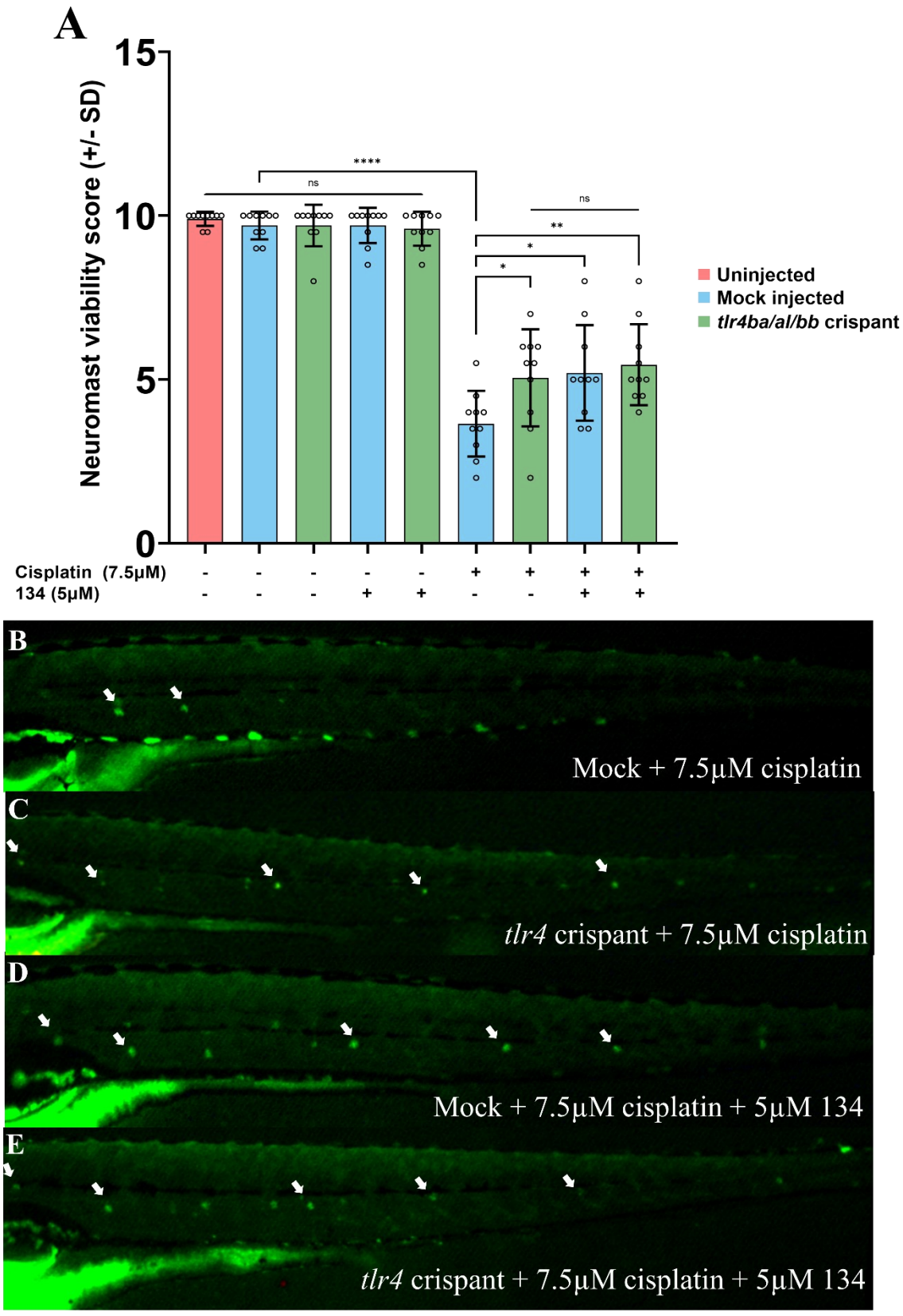


Figure 3. 9 *Tlr4 crispant* mutant larvae are less susceptible to cisplatin induced ototoxicity. Both uninjected and mock injected larvae show a significant decrease in neuromast hair cell survival after treatment with cisplatin using both DASPEI (A) and YO-PRO1 (B) staining. The *Tlr4 crispant* larvae had their *tlr4* homologs mutated, protecting them from cisplatin-induced ototoxicity. Their neuromasts showed an increase in viability (A) and number of hair cells (B) compared to the mock and uninjected cisplatin treated groups. Larvae were mutated in their *tlr4* homologs using CRISPR-cas9 genome editing. Larva were injected at the one cell stage with either gRNA sequences targeting the three zebrafish tlr4 homologs or Cas9 protein alone (mock). Larvae were grown to 6 dpf when they were treated with cisplatin for 20 hours. N = 10 for each group. ****$p<0.0001$, ns = not significant by one way ANOVA and Tukey's multiple comparison test.


A
Neuromast viability score (+/- SD)
15
10
5
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ns
ns
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*
*
Uninjected
Mock injected
tlr4ba/al/bb crispant
Cisplatin (7.5μM)
134 (5μM)
B
Mock + 7.5μM cisplatin
C
tlr4 crispant + 7.5μM cisplatin
D
Mock + 7.5μM cisplatin + 5μM 134
E
tlr4 crispant + 7.5μM cisplatin + 5μM 134

Figure 3. 10 Zebrafish TLR4 homologs are required for the otoprotective action of the TLR4 antagonist 134. A) Derivative 134 is otoprotective in wildtype (mock-injected) larvae but not in *Tlr4 crispant*s. Cisplatin treatment significantly reduced mock injected larval neuromast hair cell viability, which was recovered by mutation of tlr4 homologs by CRISPR-cas9. Mock injected larva co-treated with cisplatin and 134 showed a similar level of protection as the crispant mutants. Crispants co-treated with 134 showed a slight increase in neuromast hair cell viability in comparison to crispants treated with cisplatin alone. 7dpf Mock injected larvae treated with cisplatin shows ablation of caudal posterior lateral line neuromasts and low fluorescent intensity of rostral neuromasts (B). *Tlr4 crispant* larvae have a reduced amount of ototoxicity from cisplatin treatment resulting in an increased fluorescence from lateral line neuromasts (C). Similarly, mock injected larvae recover fluorescence when co-treated with 134 resulting in increased fluorescence and abundance of visible posterior lateral line neuromasts (D). However, TLR4 antagonist 134 adds no further protection when co-treated with cisplatin on *Tlr4 crispant* larva, which is visible by no increase in neuromast fluorescent intensity in comparison to *Tlr4 crispant* larva treated with cisplatin alone (E). Larvae were mutated in their *tlr4* homologs using CRISPR-cas9 genome editing. Larva were injected at the one cell stage with either gRNA sequences targeting the three zebrafish tlr4 homologs or Cas9 protein alone (mock). Larvae were grown to 6 dpf when they were pre-treated for one hour with 134 followed by co-treatment of with cisplatin. ****$p<0.0001$, **$p<0.0021$, *$p<0.0332$, ns = not significant by one way ANOVA and Tukey's multiple comparison test. N = 10 for each group.

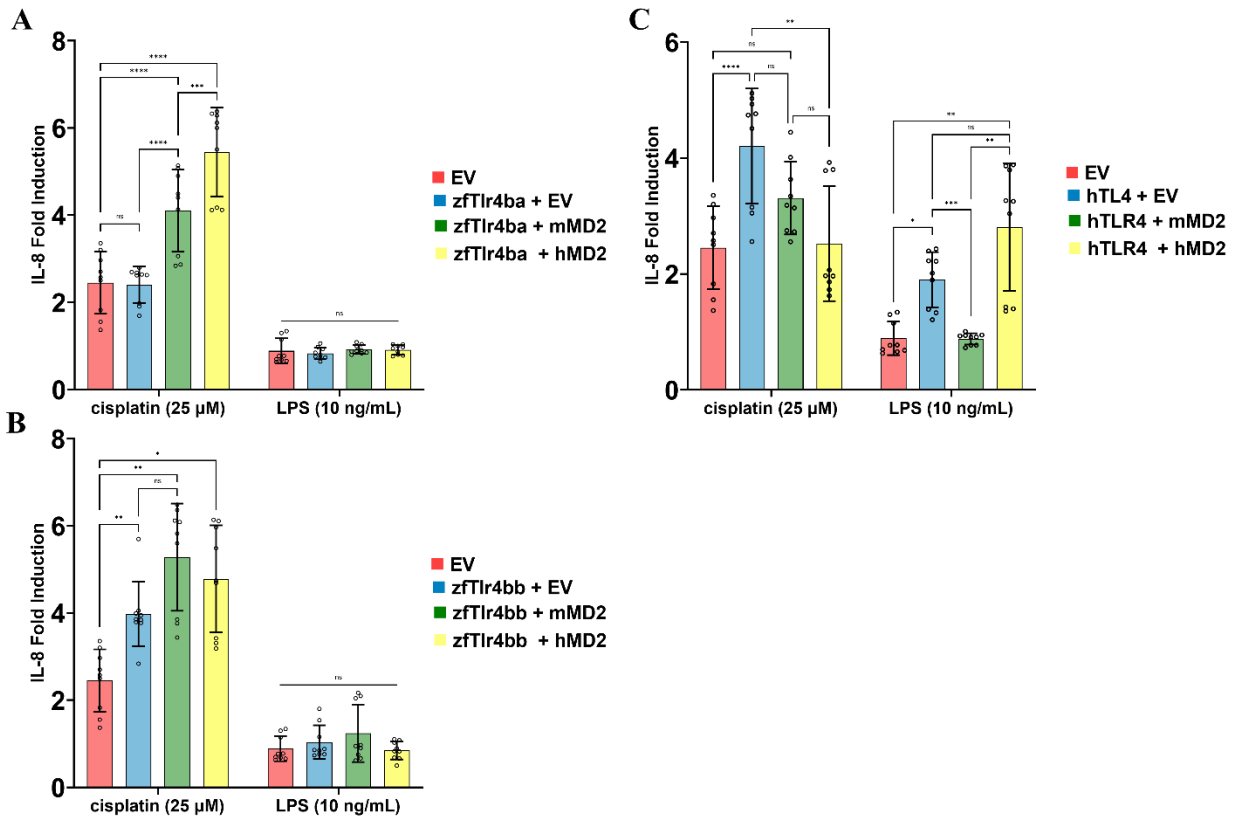

Figure 3. 11 HEK293T cells transfected with zebrafish Tlr4ba and Tlr4bb are activated by cisplatin, but not LPS. Stimulation of mammalian TLR4 by LPS results a release of a range of type 1 interferons and pro-inflammatory cytokines including IL-8 which is a common measure of TLR4 activation. HEK293 cells lacking human TLR4 (hTLR4) but transfected with Tlr4ba and Tlr4bb show increased secretion of IL-8 when treated with 25µM cisplatin, but do not when treated with 10 ng/mL of LPS. Cells transfected with hTLR4 treated with either cisplatin (25µM) or LPS (10 ng/mL) show increased IL-8 release compared to cells transfected with the empty vector alone. A) Stimulation of IL-8 release by Tlr4ba requires MD-2. Cells transfected with Tlr4ba alone show no increase in IL-8 secretion in comparison to those transfected with an empty vector alone. However, cells co-transfected with Tlr4ba and mouse (mMD2) or human (hMD2) MD-2 show an increase in IL-8 secretion in comparison to cells transfected with Tlr4ba

and the empty vector. Furthermore, the Tlr4ba-hMD-2 complex allows for increased IL-8 secretion in the presence of cisplatin in comparison to cells transfected with the mMD-2 homolog. B) Stimulation of IL-8 release by Tlr4bb does not require MD-2. Cells co-transfected with Tlr4bb and the empty vector, mouse (mMD2) or human (hMD2) MD-2 show an increase in IL-8 secretion in comparison to cells transfected with the empty vector alone. C) hTLR4 is activated by both cisplatin and LPS. Cisplatin and LPS stimulated a significant increase of IL-8 release from HEK293T cells transfected with hTLR4 alone compared to cells transfected with the empty vector only. Co-transfection with either mMD-2 or hMD-2 did not significantly increase IL-8 secretion during cisplatin treatment. Co-transfection of hMD-2, but not mMD-2 and hTLR4 increased IL-8 secretion during LPS treatment compared to cells transfected with hTLR4 alone. IL-8-fold induction was calculated based on the baseline cell secretion of IL-8 without treatment. N = three independent experiments for all treatments. ****$p<0.0001$, ***$p<0.0002$, **$p<0.0021$, *$p<0.0332$, ns = not significant by two-way RM ANOVA and Šídák's multiple comparisons test. This data was generated and provided with permission from Tracy Lee.

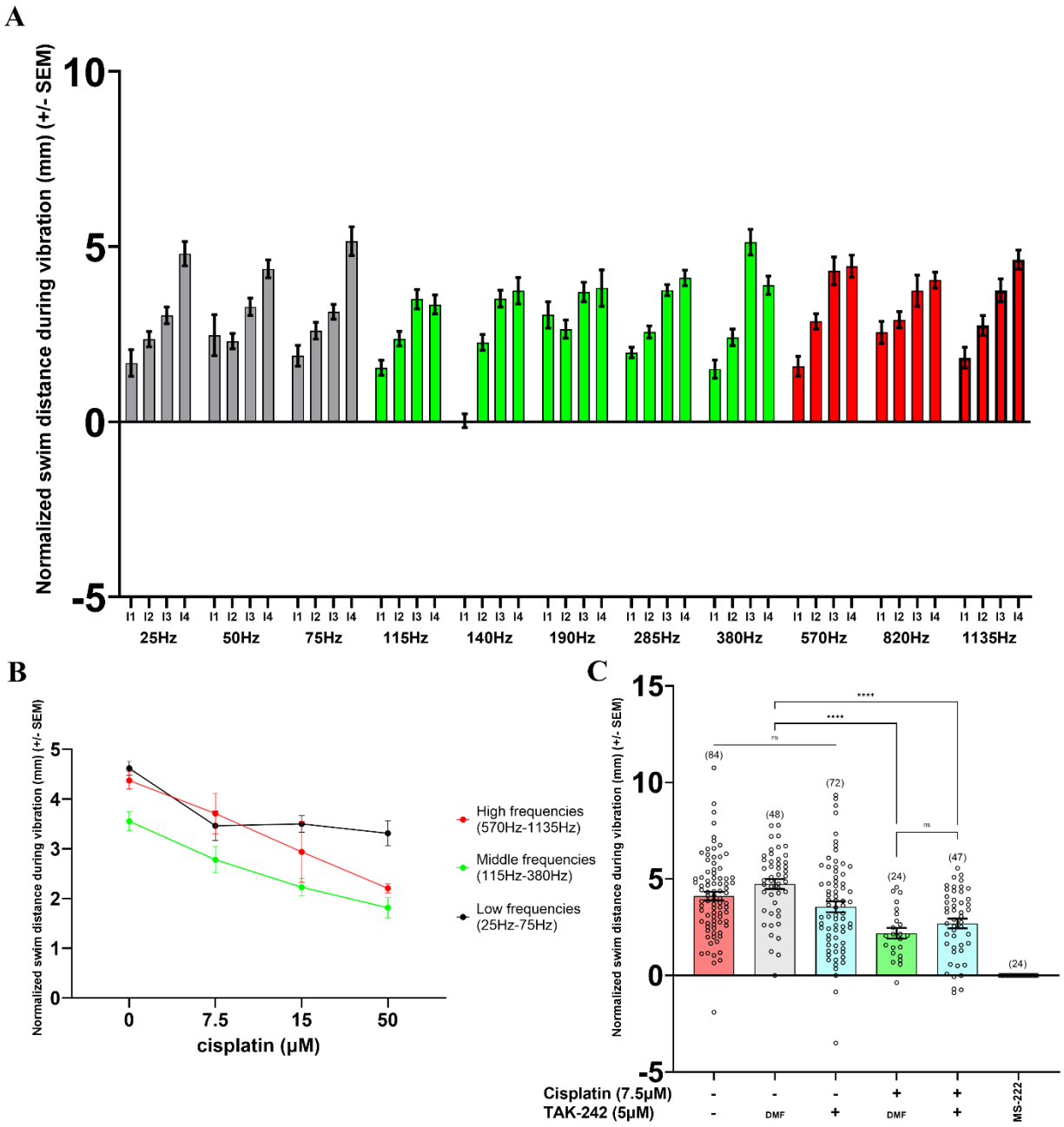

Figure 3. 12 The zebrafish startle response is dependent on vibrational intensity and is reduced in response to cisplatin induced ototoxicity. A) A range of 11 different frequencies were delivered at four vibration intensity levels with I1 being the lowest intensity and I4 being the greatest, with each step doubling in intensity. Larval swim distance was tracked during each vibration and normalized to the baseline level of movement for each individual larva. The mean

normalized distance of three vibration trials at each vibration intensity and frequency was taken for each larva. Bars represent the mean normalized startle distance of at least 72 larvae (from three independent experiments). At all frequencies, larval startle distance increased with each increase in vibrational intensity. B) Larvae were treated with a range of cisplatin concentrations for 20 hours prior to startle response distance measurement. Cisplatin decreases the functionality of lateral line neuromasts by inducing hair cell death. No matter the frequency, as cisplatin concentration increased, distance traveled during the startle response decreased. Frequencies were grouped into high (1135Hz, 820Hz, 570Hz), middle (380 Hz, 285 Hz, 190Hz, 140Hz, 115Hz) and low (75Hz, 50Hz, 25Hz). The mean startle distance of all frequencies within each group was taken at each cisplatin concentration. C) When treated alone, DMF nor TAK-242 alone had any significant effect on startle response distance in comparison to the untreated group. Cisplatin induced hair cell death, which significantly reduced startle response sensitivity, which is reflected in a reduction in the distance moved by the larva. The reduction in larval sensitivity not affected by addition of TAK-242 indicating no improved neuromast hair cell function. Larvae were pre-treated for 1 hour with either DMF (vehicle) or TAK-242, followed by a 20-hour incubation with or without cisplatin. The greatest intensity vibration (I4) was implemented for both panel B and C. Vibrational trials in panel C were performed at 285Hz. Larvae anesthetized in MS-222 were used as a control for tracking of larval movement. Bracketed numbers above each bar represents the total number of larvae in each group. ****$p<0.0001$, ns = not significant by one way ANOVA and Tukey's multiple comparison test.

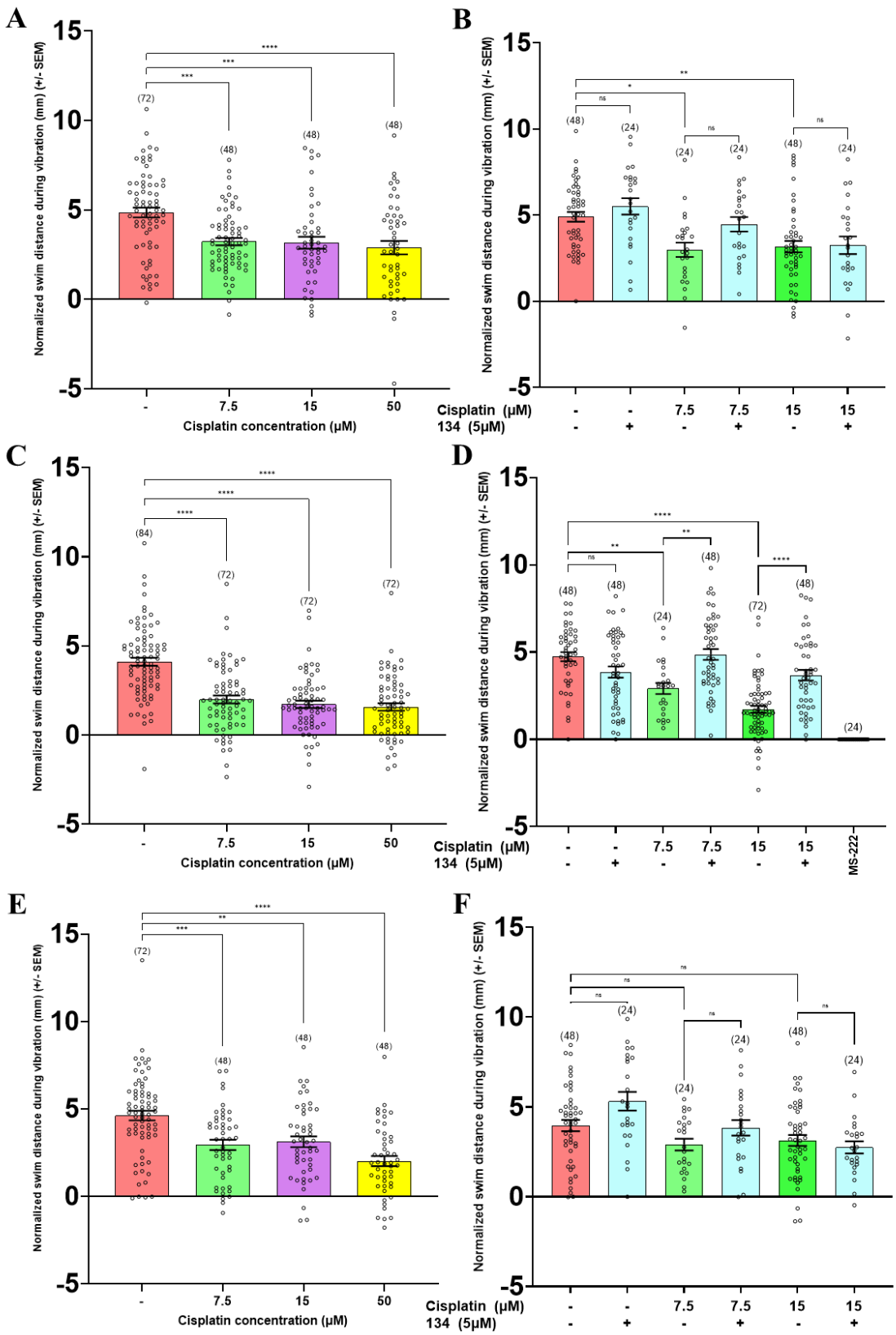
A
B
C
D
E
F
Normalized swim distance during vibration (mm) (+/- SEM)
Cisplatin concentration (μM)
Cisplatin (μM)
134 (5μM)
MS-222

Figure 3. 13 Novel tlr4 antagonists rescue hair cell function from cisplatin-induced ototoxicity. Cisplatin treatment results in hair cell death within the neuromasts of larva, resulting in a reduced sensitivity to changes in water movement. Increasing the concentration of cisplatin decreased the startle response distance of wildtype larvae after vibrations at 75Hz (A), 285Hz (C), and 1135Hz (E). 134 did not measurably alter the wildtype startle response at various frequencies. B & F) Although not significant, at 75Hz (B) and 1135Hz (F) 5μM 134 increased the startle response distance when applied with 7.5μM cisplatin. D) At 285Hz 5μM 134 fully recovered startle response distance when applied with 7.5 and 15μM cisplatin. Larvae were treated with a range of cisplatin concentrations for 20 hours prior to startle response distance measurement at different frequencies. The greatest intensity vibration was implemented for all Figures shown. Bracketed numbers above each bar represents the total number of larvae in each group. ****$p<0.0001$, ***$p<0.0002$, **$p<0.0021$, *$p<0.0332$, ns = not significant by one way ANOVA and Tukey's multiple comparison test.

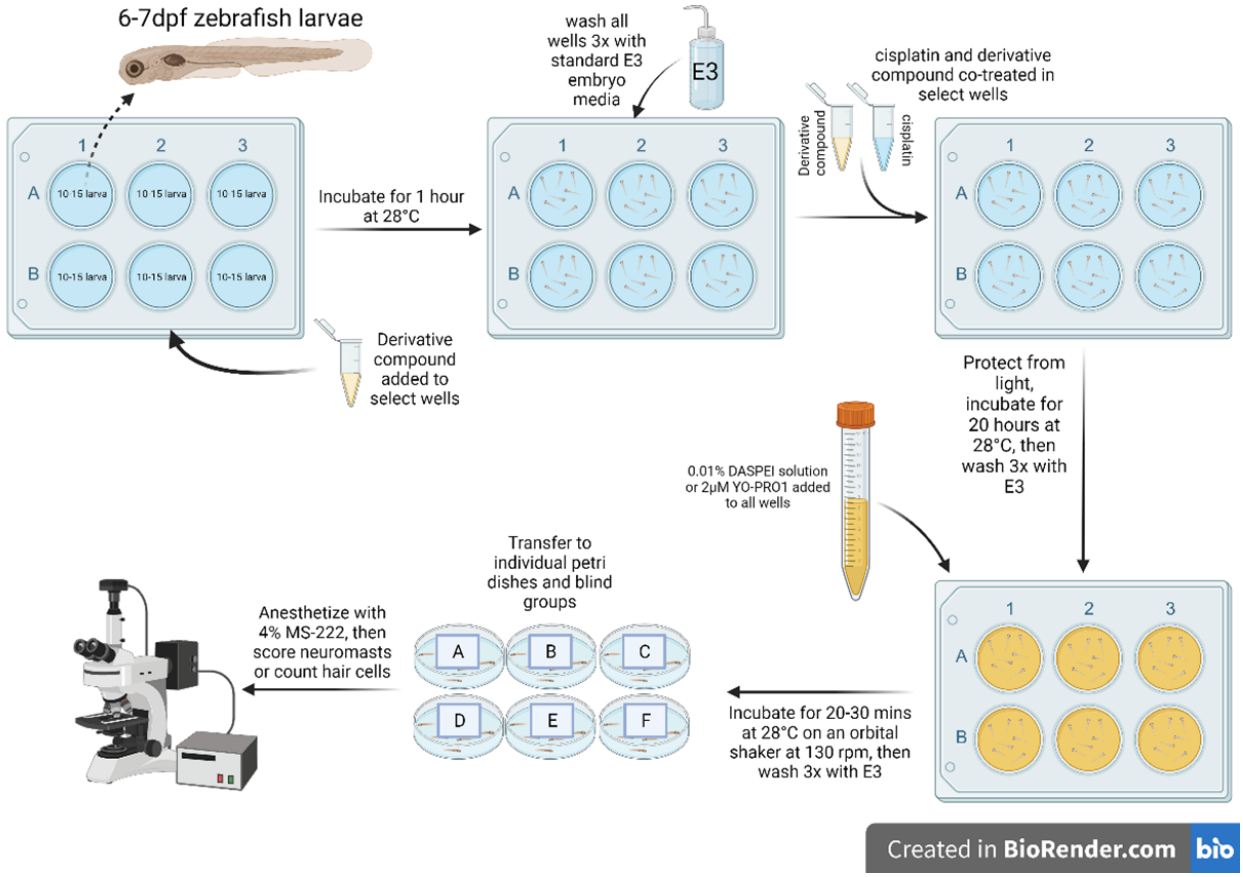

Figure 3. 14 Zebrafish larvae drug incubation and neuromast staining & quantification methodology. 10-15, 6–7-day post fertilization (dpf) zebrafish larvae are transferred into each well of a labeled 6-well plate. If a derivative was being tested, it was applied as a pre-treatment for 1 hour at 28°C. Wells were washed three times using standard E3 media and larvae were then co-treated with cisplatin and the derivative of choice, wrapped in foil, and incubated for 20 hours at 28°C. To quantify neuromast survival/health, wells were washed three times and larvae were incubated in a 0.01% DASPEI solution or a 2µM YO-PRO1 solution for 20 minutes or 30 minutes respectively. Groups of larvae were transferred into labeled petri dishes, which were then relabelled to blind the observer to the treatments. Larva were anesthetized in 4% MS-222 and scored under a fluorescent dissecting microscope. Five posterior lateral line neuromasts that are readily identifiable between individuals were selected in each larva for consistent

scoring/hair cell counting between experiments. Each neuromast stained with DASPEI was scored on a scale of 0-2 based on the fluorescent intensity, with a high score corresponding to a greater fluorescence and greater neuromast health (Chowdhury et al., 2018). Each neuromast stained with YO-PRO1 had all the hair cells counted for each of the five selected neuromasts. All five neuromast scores and hair cell counts were summed for each larva, and this score was plotted as a raw data point (dot) on the supplied graphs. Created with BioRender.

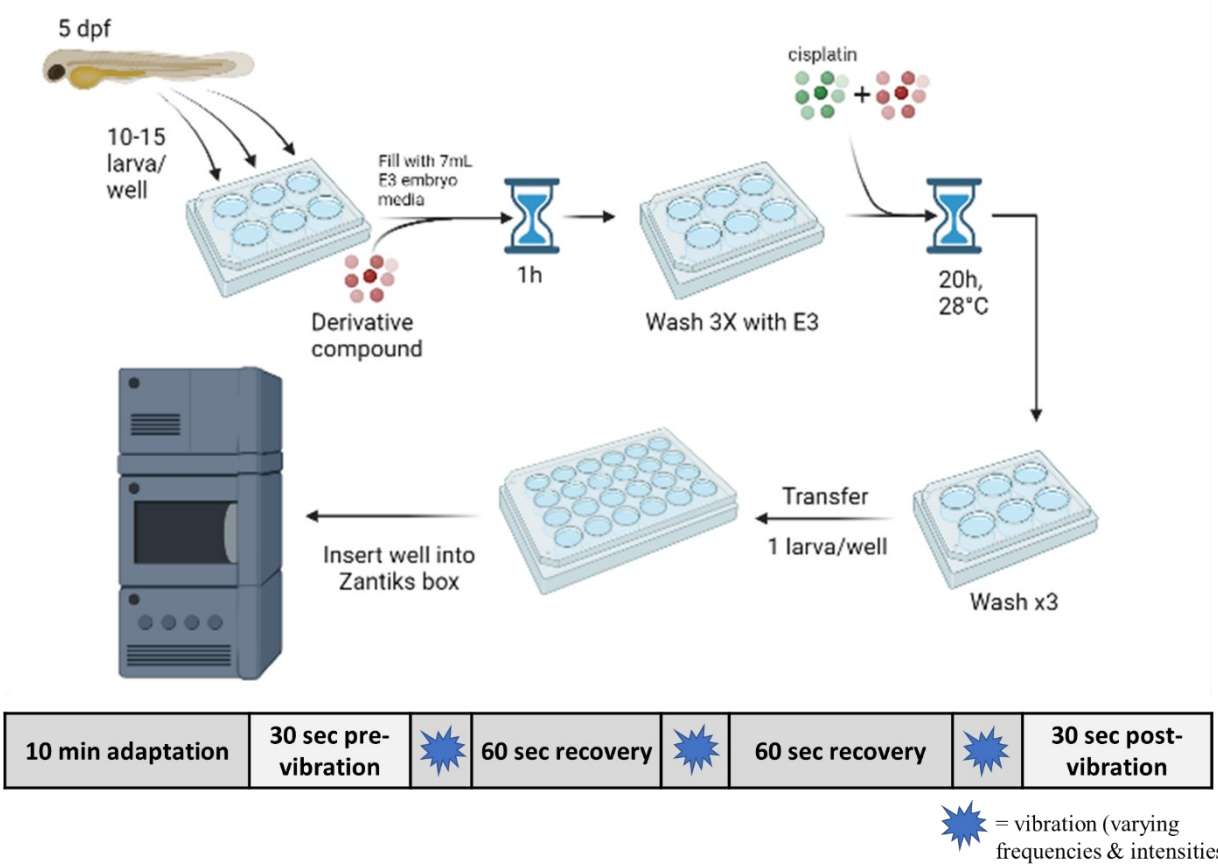

Figure 3. 15 Behavioural methodology for examining the startle response as a measure of zebrafish neuromast hair cell function following cisplatin insult. The startle response is an escape/avoidance behaviour elicited by external stimuli. As cisplatin ablates neuromast hair cells, larva will become less sensitive to vibrations within the water, producing a reduced startle response. TLR4 antagonists ameliorate cisplatin induced hair cell death, allowing for the intensity of the response to recover. Wildtype zebrafish larvae were grown to 5-6dpf at a density of <50 per petri dish. 26 zebrafish larvae were split between two wells, allowing the testing of 3 treatments per 6-well plate. If a derivative was being tested, the corresponding wells were pre-treated for 1 hour at 28°C. Wells were washed three times using standard E3 media and larvae were then co-treated and incubated wrapped in foil with cisplatin and the derivative of choice for 20 hours at 28°C. Individual larvae were transferred into separate wells of a 24-well plate, which

was transferred directly into the Zantiks Box. The Zantiks Box maintains the fish at a constant temperature of 28°C was programmed to administer vibrational stimuli equivilent to sound at varying frequencies and 4 intensities. A 10 minute and 30 second adaptation period was followed by a 1 second vibration, and a 60 second recovery period to prevent habituation. The cycle (minus the 10-minute adaptation period) was repeated three times (3 vibration trials) with the last vibration being followed by a 30 second post-vibrational period. The larvae movement was tracked and binned into 1 second values for the entire duration of the experiment. Larval movement during each vibrational trial was normalized by subtracting the distance during the vibration from the average baseline distance moved during the 30 second period prior to the respective vibration trial. Created with BioRender.

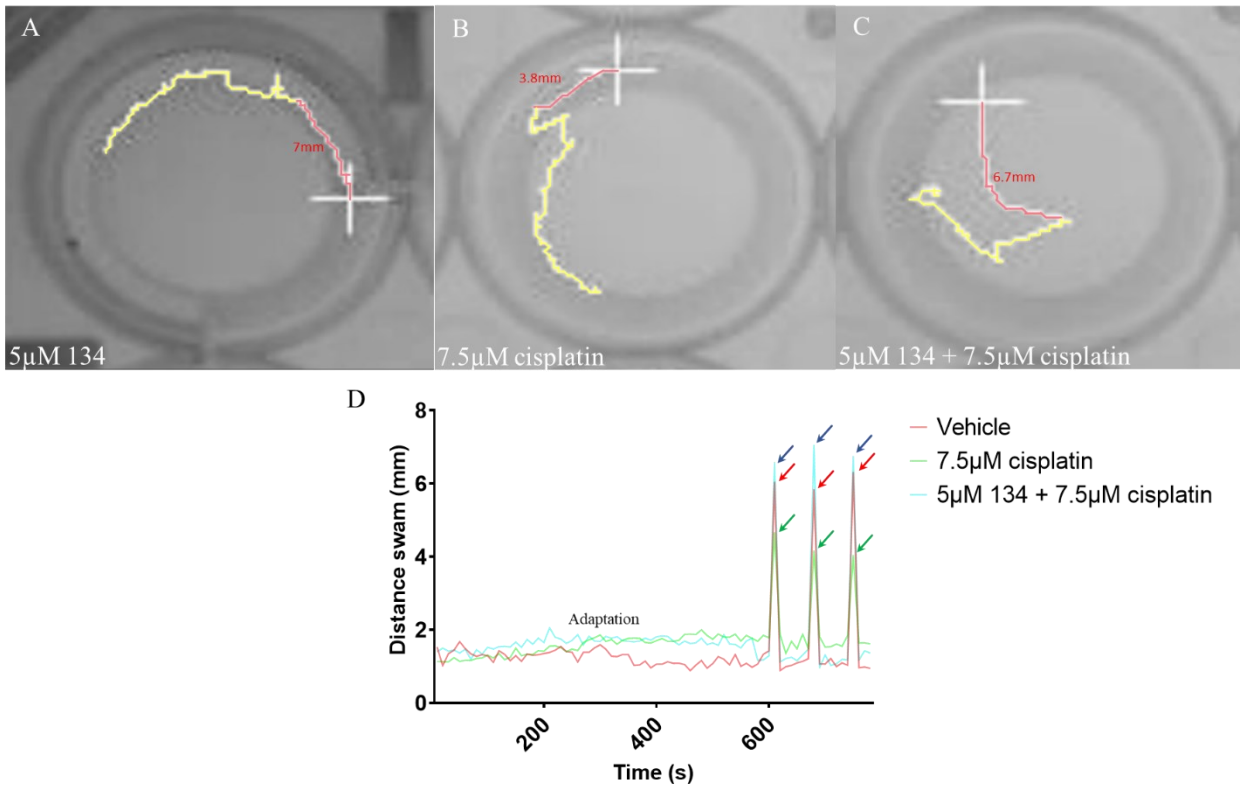

Figure 3. 16 Movement of 6dpf larvae demonstrates the recovery in startle response distance following treatment with derivative 134. A-C) Exemplar movement traces of individual larvae. Cisplatin induces hair cell death within the larvae resulting in a reduced sensitivity to vibrational stimuli and a reduced startle response distance (B). The addition of 5μM 134 improves the functionality of hair cells under assault from cisplatin, allowing increased sensitivity to vibrational stimuli and movement (C), showing a similar movement distance as 134 alone (A). The yellow line shows 5 seconds of baseline movement prior to the first vibration stimulus. Red lines denote the movement of the larvae during the 1 second vibration, with the distance written in red directly beside. D) Time course of an experiment showing the adaptation period and three individual vibrational trials at 285Hz. Co-treatment of derivative 134 with cisplatin increased the peak distance the larva moved in comparison to cisplatin treatment alone. The mean movement of 24 larvae was binned into 10 second periods except for the movement

during the 1 second vibration, which was plotted explicitly. The arrows point to the peak distance during the vibration for the vehicle (red), 7.5μM cisplatin (green), and 5μM 134 + 7.5μM cisplatin (blue) groups.

Table 3. 1 Summary of the effects of TLR4 antagonism by TAK-242 and its synthetic derivatives when applied with cisplatin and LPS in vitro and in vivo. HEI-OC1 (murine outer hair cell line) cells were pretreated with 4μM TLR4 inhibitors before co-treating with 20μM cisplatin or 10ng/mL LPS. TAK-242 derivative 132, 134, 136, and 138 showed selective inhibition against cisplatin, but not LPS in vitro. Derivatives 134, 136, 154, 156, 166 and 168 showed protection from cisplatin induced neuromast hair cell death in larval zebrafish. Only derivative 134 and 136 provided protection against cisplatin toxicity both in vivo and in vitro, while having no effect on LPS activation of TLR4 in vitro. However, 136 showed toxicity to hair cells both in vitro and in vivo. Green arrows represent protection of hair cells from CIO both in vitro and in vivo. Grey bars represent no action on when applied to hair cells both in vitro and in vivo. Red arrows represent toxicity to neuromast hair cells in larval zebrafish. Astrix represent toxicity to larval zebrafish outside of toxicity to neuromast hair cells.

	Cells + cisplatin	Cells + LPS	Zebrafish + cisplatin
TAK-242			
120			
132			
134			
136			* 
138			* 
150			* 
154			
156			
158			* 
162			
164			* 
166			* 
168			*
170			

Chapter 4. GENERAL DISCUSSION AND FUTURE DIRECTIONS

This thesis provides evidence that larval zebrafish respond to heavy metals through Tlr4, and that this response can be used as a model for chemical inhibition of Tlr4 to reduce CIO. A direct interaction of zebrafish Tlr4 homologs with metal ions is still to be determined, but the absence of Tlr4 homologs decreases metal toxicity to neuromast hair cells in vivo. Additionally, their presence promotes IL-8 release after nickel and cisplatin treatment in cell culture. Below, I will explore some of the remaining questions regarding Tlr4, the TAK-242 derivatives, and their role in mitigating CIO.

4.1 Speculations on zebrafish Tlr4 ligands

The mammalian TLR4 ligand, LPS, has been shown not to signal through zebrafish Tlr4 homologs (Sepulcre et al., 2009; Sullivan et al., 2009). Furthermore, there is no known homolog of CD-14 reported in zebrafish, which is known to be a crucial costimulatory molecule for TLR4 activation in response to LPS (Loes et al., 2021). Together, with the low sensitivity of Tlr4 homologs to LPS, this suggests that other ligands are more suited for this receptor in zebrafish (Berczi, Bertok, & Bereznai, 1966). Metals are known to directly bind to TLR4 in primates and only require the co-receptor MD-2 to mediate its downstream activation (Oblak, Pohar, & Jerala, 2015; Rachmawati et al., 2013; Raghavan et al., 2012; Schmidt et al., 2010). The presence of MD-2 (*ly96*) homolog in the zebrafish genome gives support to metals acting as a suitable ligand for Tlr4 homologs in zebrafish (Loes et al., 2021).

While being functionally disparate, zebrafish Tlr4 homologs still share many similarities with mammalian TLR4. For instance, their overall structure is conserved, displaying an ectodomain, transmembrane domain, and intracellular (TIR) domain (Figure 2.2). They also

express homologs for almost every adaptor molecule, as well as many signalling molecules such as Myd88, Ikkap (IKK complex associated proteins), Trif, Irak4, Irak1, all Traf family members, Rip1, all IRF family members, Nfκβ, and more (Fan et al., 2008; Li et al., 2017; Liu et al., 2010; Ordas et al., 2011; Phelan, Mellon, & Kim, 2005; Srivastava et al., 2017; Stein et al., 2007). One signalling adaptor not yet found within zebrafish is TRAM, an adaptor responsible for inducing TRIF activation and subsequent type 1 IFN release within mammals (Sullivan et al., 2007). However, it has been determined that Trif activation and IFN release within zebrafish still occurs through other pathways (such as Tlr3, Tlr22), suggesting Tlr4 mediated Trif activation may be absent, or occurs through a different mechanism from mammals (Candel et al., 2015; Li et al., 2017; Sullivan et al., 2007). Other proinflammatory cytokines released from mammalian TLR4 activation are also conserved as homologs within zebrafish, including, but not limited to: IL-6, TNF-α, and IL-1β (Campos-Sánchez & Esteban, 2021; Li et al., 2021; Srivastava et al., 2017). An array of ligands has been reported to induce inflammatory responses within zebrafish but fail to show their direct involvement with Tlr4. Being capable of demonstrating the binding of Tlr4 and the initiation of the associated proinflammatory response is imperative to studying its function and evolutionary origins.

When speculating on the function of zebrafish Tlr4 and its ligands, it is important to determine the response of zebrafish to the primary ligand of TLR4 in mammals, LPS. Loes et al. determined that Tlr4ba can elicit a response to LPS, but only in the presence of mammalian CD14 and high concentrations of LPS (>200ng/mL) (2021). Human TLR4 is highly sensitive to LPS and capable of being activated at concentrations less than 0.02ng/mL, even though experimental concentrations are generally equal to ~10ng/mL (Cohen et al., 1995; Huang et al., 2012; Schwarz et al., 2014; Sepulcre et al., 2009; Van der Poll et al., 1994; West & Koons,

2008). Similarly, other studies have used high levels of LPS on zebrafish to produce disease models and examine protein function (Sellaththurai et al., 2023; Wolińska-Nizioł et al., 2022; Xiao et al., 2022). Our results contrast these findings as Tlr4 homologs transfected in cells in vitro failed to respond to LPS (Figure 2.9; Figure 3.11).The responses observed in these studies may be due to contamination with peptidoglycan, a highly immunogenic substance for fish immune cells (MacKenzie et al., 2010). What ever the case may be, due to the high conservation of inflammatory genes and proteins, these studies have demonstrated that zebrafish can be used as an apt model for inflammatory responses. However, the influence of Tlr4 to these findings requires further investigation. Using genetic alteration of Tlr4 through CRISPR-cas9, as demonstrated in our studies above, can provide further insight into Tlr4s contribution to these disease models as well as its interaction with other inflammatory pathways.

Beyond TLR4, mammals can respond to LPS through cytosolic proteins called caspases (Shi et al., 2014). In vertebrates, caspases are responsible for promoting a wide range of functions, including inflammatory responses, apoptosis, cell proliferation, differentiation, migration, survival, tissue repair, and regeneration (Spead et al., 2018). Mammalian inflammatory caspases can be characterized by a CARD (N-terminal caspases recruitment domain) domain. CASPASE-4 and CASPASE-5 in humans, and Caspase-11 in mice, can use this CARD domain to bind intracellular LPS leading to its activation, cleavage of gasdermin D, and subsequent cellular pyroptosis (a mode of inflammatory cell death) (He et al., 2015; Shi et al., 2014; Yang et al., 2018). However, zebrafish inflammatory Caspases (-1, -19a, 19b) contain an N-terminal pyrin domain (PYD) in place of the CARD domain in mammals (Spead et al., 2018). Despite this difference, Caspy2 in zebrafish (also known as Caspase-19a) has recently been observed to use this PYD domain in a similar manner to the CARD domain, binding LPS,

inducing inflammasome formation, and pyroptosis (Spead et al., 2018; Yang et al., 2018). Spead et al. demonstrated that Caspy2 is involved in resistance to bacterial sepsis and induced an increase in the inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, IL-10, and IFN-γ following infection (2018). Furthermore, Chen et al. demonstrated that following gram-negative bacterial infection, Caspy2 induced pyroptosis in neutrophils, which facilitated the release of neutrophil extracellular traps (NETs), leading to bacterial clearance (2021). To our knowledge, this is the only pathway shown to directly interact with LPS in zebrafish and could possibly represent a compensatory pathway for the lack of LPS sensitivity observed by zebrafish Tlr4.

Characterizing zebrafish Tlr4 and its downstream signalling is difficult without a known primary ligand to activate the receptor. In chapter 2, we have provided evidence that metals mediate zebrafish Tlr4 activation. This interaction could be used to help elucidate the mechanism behind zebrafish Tlr4 binding and its downstream functions. Similar to our cell culture experiments, previous studies have demonstrated an increase in gene and/or protein expression of Tlr4, adaptor/signalling molecules, and proinflammatory cytokines in response to different potential ligands (Li et al., 2021; G.-p. Liu et al., 2018; Srivastava et al., 2017; K. Wang et al., 2023; Wen et al., 2022; Xu et al., 2021). To our knowledge, only one ligand, peroxiredoxin 1, has been shown to directly bind zebrafish Tlr4ba, suggesting a role of zebrafish Tlr4 in DAMP recognition (G.-p. Liu et al., 2018). While we have provided evidence that metals signal through Tlr4 to induce responses such as cell damage and proinflammatory cytokine release, no direct interactions were determined. Therefore, future studies should first establish a direct binding interaction between zebrafish Tlr4 and metals. Due to the unique nature of this ligand, assays normally used in determination of receptor-ligand interactions, such as co-immunoprecipitation, cannot be used. However, other methods to examine the interaction between a receptor and

ligand exist, including, but not limited to: affinity chromatography, microscale thermophoresis, equilibrium dialysis, and solid phase microextraction (Chen et al., 1993; Domingo et al., 2023; Musteata & Pawliszyn, 2005; Videen et al., 1992). Once an interaction is determined, subsequent studies should aim to examine expression levels of adaptor and signalling proteins as well as transcription factor activity to help characterize the zebrafish Tlr4 response.

4.2 Zebrafish in the evolution of TLR4

Due to the vital function of the TLR4 pathway in inflammation within mammals, it is of particular interest to examine the origins of this pathway to further understand this important pathway in humans. TLR4 is known not only to respond to PAMPs, but is a critical mediator of inflammation in response to endogenous damage signals. The importance of Toll-like receptors to DAMPs can be exemplified by the first observed Toll receptor in Drosophila, an ancient organism that was believed to diverge from vertebrates ~830 million years ago (Gu, 1998). Toll was found to be responsible for recognition of the ligand Spatzle, an endogenous protein released during fungal infection (Valanne, Wang, & Rämet, 2011). Furthermore, the response of TLR4 to DAMPs can vary in which co-receptors are required for activation, which is different in comparison to LPS recognition that requires both MD-2 and CD14. Fish appear to lack a homolog of CD-14, yet have been observed to bind and signal through peroxiredoxin 1, a ubiquitously expressed DAMP (He et al., 2019; G.-p. Liu et al., 2018).

The observations above suggest that TLR4 may have originally evolved as a receptor for endogenous ligands and later acquired co-receptors enabling its recognition of LPS and other PAMPs. The aquatic environment of fish, which supports a greater abundance of microorganisms in comparison to land, may have impeded the evolution of Tlr4 towards PAMP recognition and even result in the loss of this receptor in some species (Falkowski & de Vargas,

2004; Iliev et al., 2005; Roach et al., 2005; Watson et al., 1977). Another related explanation posits that the gain of CD-14 was never advantageous within fish, not only due to their environment, but also because of the selective pressures from ligands not requiring this co-receptor. This explanation supports our findings that Tlr4 is principally involved within the inflammatory response of zebrafish to metals, as well as the metal based chemotherapeutic cisplatin (Figure 2.14; Figure 3.9). As previously mentioned in chapter 2, metals are observed to act as inflammatory agents within zebrafish (Brun et al., 2018; Chen et al., 2019; T. Wang et al., 2015), but they also induce these affect in a broad range of other species such as mice, chickens, turtles, and frogs (Arienzo, 2023; Habotta et al., 2022; Jayawardena et al., 2016; Vennegaard et al., 2014). This could suggest an ancient origin of metal ion sensing by the innate immune system, and more specifically, Tlr4.

4.3 The inhibition of Tlr4 ameliorates CIO

TLR4 was recently observed to mediate CIO, which opens the possibility of novel therapeutic opportunities (Babolmorad et al., 2021). Moreover, our results in Tlr4 crispant mutants supported these findings, displaying reduced neuromast toxicity from cisplatin (Figure 3.9). We provided evidence of the TAK-242 derivative 134 reducing cisplatin cytotoxicity to neuromast hair cells, and that this effect was principally mediated through inhibiting Tlr4 (Figure 3.4; Figure 3.9). The specificity of this derivative is exemplified using mutant Tlr4 crispants, which on their own showed reduced cytotoxicity from cisplatin, but demonstrated no further increase in protection from 134 (Figure 3.9A).

4.3.1 Effects of cisplatin on zebrafish

One of cisplatin's most well studied effects within larval zebrafish is its ability to induce hair cell damage within both the lateral line and inner ear (Ou, Raible, & Rubel, 2007; Wertman

et al., 2020). We observed dose-dependent hair cell death in response to cisplatin treatment using two different vital fluorescent dyes, DASPEI and YO-PRO1 (Figure 3.3A&B). DASPEI is known to selectively stain neuromasts based on mitchondria potential and therefore fluoresces proportionately based on cell viability (Henshall et al., 2009; Jensen & Rekling, 2010). YO-PRO1 is a DNA stain, however, its uptake mechanism by neuromasts is still unknown. DASPEI is useful as it gives an overall picture of neuromast viability based fluorescent intensity, while YO-PRO1 allows for the viewing of individual hair cells in response to cisplatin. Largely, the results from these dyes mirrored one another, increasing experimental validity.

However, one difference in their results was found in how they respond to high concentrations of cisplatin. DASPEI fluorescence was completely ablated in response to high concentrations (Figure 3.3A), while YO-PRO1 fluorescence persisted within some cells (Figure 3.3B). It is possible that during early cell death, the DNA that remains within the cytoplasm of cells is bound by YO-PRO1, giving the illusion of a live cell (Elmore, 2007). However, further study is required on the mechanism behind the uptake of YO-PRO1 in neuromasts and other selective fluorescent dyes to clarify this result. In mammals, due to its relatively large size, YO-PRO1 is unable to enter the membrane of live cells. Only during cell death does the membrane become permeable to the stain, which enters through P2X7 receptor and selectively binds the DNA of dying cells (Fujisawa et al., 2014). Therefore, to examine the mechanism of YO-PRO1 uptake by neuromast hair cells, future studies should focus on membrane receptors and channels that may carry out similar functions.

On top of the effects cisplatin exerts on the inner structures of cells, it also damages the cilia of neuromast hair cells (Kim et al., 2008). Zebrafish neuromast hair cells contain both kinocilia and stereocilia, both of which are required for their proper function (Kindt, Finch, &

Nicolson, 2012). A study done by Comis et al. found that following cisplatin treatment, the stereocilia of the outer hair cells from the cochlea of a guinea pig were disordered and fused (1986). This is consistent with our findings that lateral line hair cell cilia became fused and disorganized when larval zebrafish were treated with high concentrations of cisplatin (Figure 3.3E-H). Both the disorganization and fusing of cilia may also contribute to the reduced function observed within the neuromast hair cells, which may help explain some of our behavioural data (Figure 3.12B).

4.3.2 Toxic effects of derivative compounds on zebrafish

In the determination that 134 is the most efficacious derivative, others were disregarded due to their toxic effects on zebrafish larvae. One of the most shocking results we observed was the toxicity to neuromast hair cells when TAK-242 was treated in combination with cisplatin (Figure 3.4B). It is noteworthy that this was not observed when TAK-242 was treated on zebrafish alone. No toxic effects were observed within cells after treatment with TAK-242 and cisplatin, suggesting this effect is a specific to zebrafish (Table 3.1; Data not shown). Furthermore, other derivatives such as 136, 138, 150, 158, 164, 166, and 168 were determined to induce general toxicity within zebrafish larvae, often resulting in death (Table 3.1). Only TAK-242, derivative 120, and 132 were specifically toxic to neuromast hair cells without inducing other noticeable toxicity to the larvae. Unfortunately, these responses do not appear to be localized within a specific structural modification of TAK-242, making it difficult suggest a mechanism based on their structure (Data not shown). However, another interesting finding was that derivative 138 and 158 exhibited reduced toxicity to zebrafish when in the presence of cisplatin, which is the opposite effect we predicted would occur. This would suggest some

protective ability of cisplatin from the toxicity of these derivatives, however too little is known about the interactions of these derivatives to suggest a potential mechanism by which this occurs.

4.3.3 Efficacy of cisplatin following derivative treatment

Although the primary mechanism through which cisplatin mediates its anticancer effects is believed to be through DNA damage, many of its effects are mediated through the generation of ROS (Brozovic, Ambriović-Ristov, & Osmak, 2010; Ghosh, 2019). Inhibition of TLR4 would reduce the inflammatory response generated by cisplatin, which may further lead to a reduction in ROS generation. Although this could help reduce unwanted cell death signalled through these pathways, there is a chance the anticancer efficacy of cisplatin could be reduced as well. To study this possibility, our collaborators within the Berman lab are using zebrafish xenografts to examine reductions in the anticancer effects of cisplatin in response to these derivatives. If these derivatives prove to reduce the effectiveness of cisplatin, future studies should first examine why this would occur, as DNA damage should not be impeded. However, due to the novelty of these drugs, they may be inadvertently interacting with aquated cisplatin or interacting with other unknown targets leading to a decrease in free cisplatin concentration, decreased cisplatin permeability, increased cisplatin metabolism, increased cisplatin toxicity, etc.

4.3.4 Behavioural analysis as a functional measure of zebrafish hair cells

Behavioural experiments are a unique advantage to in vivo models that can be used to examine the cumulative phenotypic effects following experimentation with novel compounds. The involvement of lateral line hair cell inputs has been demonstrated in behavioural responses, such as the startle response, through recovery experiments (McHenry et al., 2009). Ototoxic compounds, such as neomycin and gentamicin, have demonstrated a reduction in the ability to perform behaviours such as rheotaxis and the startle response, which is likely mediated by the

reduced sensitivity of the lateral line after hair cell damage (Buck et al., 2012; X. Liu et al., 2018; Newton et al., 2023; Niihori et al., 2015; Todd et al., 2017; Wang et al., 2017). While others have demonstrated a reduced rheotactic response after cisplatin treatment (Lee et al., 2022; Niihori et al., 2015), previous attempts to displays cisplatin's ototoxic effects using the startle response has been largely unsuccessful (Buck et al., 2012). Buck et al. found that following cisplatin treatment, there was no significant decrease in auditory evoked startle response in 5dpf larvae (2012). To our knowledge, we are the first study to effectively display the dose-dependent ototoxic effects of cisplatin using a vibration-based startle response (Figure 3.12B). One reason for the difference in our results in comparison to Buck et al. could be due to the short exposure time to insufficient concentrations of cisplatin (2012). They tested responses following a 2-hour incubation with either 14µM or 100µM cisplatin, which may not have allowed for adequate hair cell damage and/or loss of function (Buck et al., 2012; Lee et al., 2022). Another difference can be found in the method to elicit a startle response, where they utilized a speaker to generate a startle response, while our assay used a motor to directly vibrate the plate and evoke the response. Finally, a minimum of 36 larvae from 3 experimental repeats were tested using only one frequency (200Hz) following cisplatin treatment, while we tested a minimum of 96 larvae from three experimental repeats per frequency following different cisplatin concentrations. Further study is required to determine the influence of these differences.

Others have demonstrated the efficacy of otoprotectants, such as ferulic acid and dexamethasone, in recovering behavioural responses following treatment with ototoxins (Cheng et al., 2023; Niihori et al., 2015). Cheng et al. demonstrated that the distance moved in response to a taping stimulus was significantly improved following ferulic acid treatment to attenuate neomycin induced hair cell death (2023). Similarly, we show that co-treatment of cisplatin and

compound 134 increased the distance moved by larvae following a vibrational stimulus (Figure 3.13D). This demonstrates the endpoint protective effects by 134 within the hair cells of the lateral line. However, although no changes in baseline behaviour were observed following treatment with 134 and/or cisplatin (Figure 3.16D; data not shown), there is a possibility that other effects beyond the lateral line are responsible for the increased swimming distance. For example, neomycin is observed to decrease larval movement, not only through hair cell death, but by muscle damage (Han et al., 2020). Cisplatin is toxic, and more specifically a neurotoxic substance, therefore it may affect the neuromuscular system and/or the brain, leading to altered responses following external stimuli. Further characterization of these derivatives, their interaction with cisplatin and the systemic effects of cisplatin on larval behaviour will help exclude these as confounding variables.

4.3.5 Insight into the effect of frequency on zebrafish behaviour

Zebrafish larvae are observed to respond to frequencies ranging from 40-1200 Hz (Bang et al., 2002; Bhandiwad et al., 2018; Lu & DeSmidt, 2013; Roberts et al., 2011; Zeddies & Fay, 2005). Buck et al. examined the startle response to a range of frequencies between 40 – 500 Hz and found no significant effect of frequency on startle distance (2012). In agreement with these results, we found that in a range of 25 – 1135 Hz, the startle response did not appear to vary, suggesting frequencies lower than 40 Hz can elicit a response (Figure 3.12). However, in contrast to this, cisplatin treatment resulted in varying affects to the startle response distance following vibrations at different frequencies (Figure 3.12B). Furthermore, we found that startle responses induced by both higher (1135 Hz) and lower (75 Hz) frequencies displayed no significant recovery in the distance after treatment with 134 and cisplatin, but the response induced within the middle (285 Hz) frequency was significantly improved (Figure 3.13B, D, F).

Interestingly, middle frequencies (115-380 Hz) induced the lowest mean startle distance, with or without cisplatin (Figure 3.12B). However, additional research is necessary to ascertain whether these findings are related in any manner. In humans, CIO is known to initially affect higher frequencies (4000-8000Hz), that can progress into lower frequencies (Chattaraj et al., 2023). Little is known on how cisplatin affects different frequencies in zebrafish within the literature, but our results suggest that this would be a valuable avenue for future study. Based on what is known on progressive hearing loss in humans, future studies should consider not only concentration, but also the length of exposure to cisplatin.

4.4 Future directions and conclusion

In addition to those mentioned above, a logical next step would be to produce a stable Tlr4ba, Tlr4al, and Tlr4bb triple knockout mutant line. This would allow for a more reliable examination into the effects of cisplatin and metals observed within our Tlr4 crispant mutants, while also corroborating and validating our findings. Since the overall role of Tlr4 within zebrafish remains elusive, these stable mutans would also allow for a more in-depth examination into the role zebrafish Tlr4 homologs play in development, immune function, survival, and more.

Following the generation of a triple knockout mutant, producing zebrafish expressing human TLR4 within their neuromast would provide important information on the effects of inhibiting human TLR4 in vivo, and provide an accurate model for future experiments using the TAK-242 derivatives. Moreover, to determine the influence endogenous signals may be contributing to activation of zebrafish Tlr4 during our metal and cisplatin assays, it would be valuable to examine Tlr4 activation in response to well known DAMPs such as HMGB1, heat shock proteins (HSP), and β-defensin 2, all of which have known homologs in zebrafish (García-

Valtanen et al., 2014; Lam, Harvie, & Huttenlocher, 2013; Yu, Wang, & Chen, 2010; Zhao et al., 2011).

One major limitation to the findings of this thesis is found in the lack of a positive control for zebrafish Tlr4 activation. Since there is currently no known primary ligand of Tlr4 reported within the literature, producing a reliable assay that demonstrates the activation of Tlr4 is difficult. We have demonstrated that hair cell toxicity can be used as a suitable proxy for the downstream effects from Tlr4 activation, but this would have benefited from a known primary ligand.

In summary, the key findings of this thesis are two-fold: first, we demonstrated that metals could mediate a proinflammatory response through zebrafish Tlr4 and its genetic modification reduces metal cytotoxicity. This finding helps provide insight into the both the evolution and function of this receptor in zebrafish. Second, we demonstrated that through genetic modification and chemical inhibition of zebrafish Tlr4, the ototoxic effects of cisplatin could be mitigated. This finding provides support to the use of TLR4 inhibitors in preventing CIO, which could afford major improvements to the quality of life in cancer patients receiving treatment, especially in children. Altogether, these findings will set the groundwork for future studies examining the involvement of metals in activating zebrafish Tlr4 homologs and their effective use in determining novel therapies for the reduction of CIO.

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