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Editorial

Use of zebrafish larvae lateral line to study protection against cisplatin-induced ototoxicity: A scoping review

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Abstract

Aim: The present review aimed to consolidate and analyze the recent information about the use of zebrafish in studies concerning cisplatin-induced ototoxicity and otoprotection.

Material and methods: The PubMed, Web of Science, and Scopus databanks were searched using the following MESH terms: zebrafish, cisplatin, ototoxicity. The identified publications were screened according to inclusion and exclusion criteria and the 26 qualifying manuscripts were included in the full-text analysis. The experimental protocols, including cisplatin concentrations, the exposure duration and the outcome measurements used in zebrafish larvae studies, were evaluated and the reported knowledge was summarized.

Results: Twenty-six substances protecting from cisplatin-induced toxicity were identified with the use of zebrafish larvae. These substances include quinine, salvianolic acid B, berbamine 6, benzamil, quercetin, dexmedetomidine, dexamethsanone, quinoxaline, edaravone, apocynin, dimethyl sulfoxide, KR-22335, SRT1720, ORC-13661, 3-MA, D-methionine, mdivi-1, FUT-175, rapamycin, Z-LLF-CHO, ATX, NAC, CYM-5478, CHCP1, CHCP2 and leupeptin. The otoprotective effects of compounds were attributed to their anti-ROS, anti-apoptotic and cisplatin uptake-blocking properties. The broadest range of protection was achieved when the experimental flow used preconditioning with an otoprotective compound and later a co-incubation with cisplatin. Protection against a high concentration of cisplatin was observed only in protocols using short exposure times (4 and 6 h).

Conclusions: The data extracted from the selected papers confirm that despite the differences between the human and the zebra fish hearing thresholds (as affected by cisplatin), the sensory cells of zebrafish and larval zebrafish are a valuable tool which could be used: (i) for the discovery of novel otoprotective substances and compounds; (ii) to screen their side effects and (iii) to extend the knowledge on the mechanisms of cisplatin-induced inner ear damage. For future studies, the development of a consensus experimental protocol is highly recommended.

Keywords

animal models, cisplatin, otoprotection, ototoxicity, zebrafish

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Introduction

Hearing loss is a widespread human sensory disability adversely affecting the communication, social isolation, depression and the quality of life of the affected persons. One of the common causes of hearing loss is the exposure to ototoxic substances such as heavy metals or ototoxic drugs, aminoglycoside antibiotics (neomycin, gentamycin), loop diuretics, and platinum-based cytostatic drugs (cisplatin, oxaliplatin, and carboplatin). The platinum-containing medications are used for the treatment of solid cancers (e.g., head and neck carcinomas, lung carcinomas, cervical carcinomas, or melanomas).[1,2] Unfortunately, the incidence of cisplatin-induced ototoxicity is high and the cisplatin-induced hearing loss is not only bilateral, progressive and irreversible but also often associated with vertigo and tinnitus.[3,4] According to the literature, children are more prone to develop hearing loss following cisplatin treatment than adults. Loss of hearing at an early age has negative psychosocial consequences, negatively affecting the development of the affected child.[5,6]

Recent research has concentrated on understanding the mechanisms of cisplatin-induced inner ear damage and on identifying anti-ototoxic substances. Since auditory cell-lines cannot substitute the mature hearing organ, animal models are used in ototoxicity research, including rats, guinea pigs, mice and zebrafish (*Danio rerio*).

Despite many differences, the zebrafish and human models share considerable similarities and zebrafish can be used to screen conditions observed in human pathologies.[7] The easy accessibility to the hearing organ, the small size and the structural and functional similarities between zebrafish and mammalian hair cells, make zebrafish a valuable animal model for studying cisplatin-induced hearing loss. The zebrafish possesses hair cells on the outside of its body in a sensory system called the lateral line. In the lateral line, mechanosensory hair cells are organized into small groups called neuromasts. Each neuromast contains 10–20 hair cells and associated supporting cells.[8–14] In zebrafish larvae, neuromasts are mature with functional hair cells by 3 days post-fertilization (dpf).[15] The physiological similarities between lateral line and hair cells of the inner ear, easy visualization of the lateral line, and full maturity by 3 days post-fertilization make the lateral line of larvae zebrafish an ideal model for screening large numbers of individual drugs and drugs combinations. The adult zebrafish are used less frequently in the ototoxicity-related studies than the larval zebrafish. There are several advantages of using zebrafish larvae in this type of research, including their permeability to small molecules, their small size and transparency, their rapid generation time and the lateral hair cell similarity to the mature hair cells in the inner ear of adult zebrafish.

Although the zebrafish model offers many advantages over the other animal models, it is essential to remember that in humans, cisplatin induces a hearing loss in the high frequencies, while in zebrafish, only the low frequencies are affected. Also, the sensory hair cells of the fish can regenerate, which a feature not observed in mammals. Therefore, only the acute ototoxicity can be studied using the zebrafish model, not matching the chronic ototoxicity that induces a permanent hearing loss in humans.[16]

The present review focused on the lateral line of zebrafish larvae and adult animals in studies of cisplatin-induced sensory hair cell loss and otoprotection. The main goal was to analyze the usefulness of the zebrafish model to identify new substances protecting against cisplatin and to explore new knowledge about the mechanisms mediating ototoxicity. The experimental protocols used in the included publications, the protective mechanisms and the adverse effects of the identified substances were evaluated.

In this scoping review, articles published between 2009 and 2020 were considered.

Methods

The present study searched for papers published between January 2009 and May 2020, using the following databanks:

- US National Library of Medicine - National Institutes of Health (PubMed);
- Scopus;
- Web of Science.

The search was restricted to publications in the English language. The keywords included the following combination of mesh terms: zebrafish AND cisplatin AND ototoxicity. Full-text articles were downloaded when the title, abstract, or keywords suggested that the study may be eligible for this

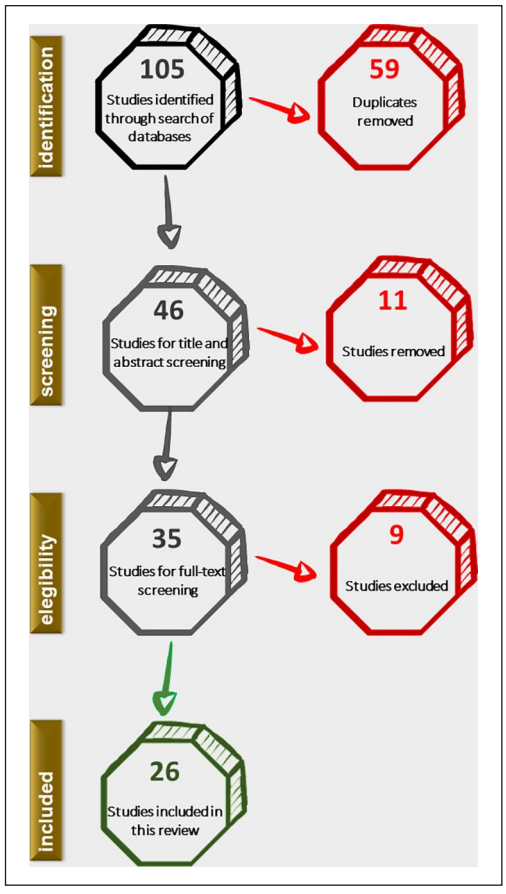

Figure 1. Flowchart of the study.

review. The selection procedure followed the inclusion and exclusion criteria summarized below (see also Figure 1).

Inclusion criteria

- articles published in the last 12 years (2009–2020)
- original research
- articles dedicated to studying molecules or compounds protecting from cisplatin-induced toxicity to sensory hair cells
- using zebrafish[1]

Exclusion criteria

- full text not available
- literature review
- lack of information about the experimental groups

After applying the selection criteria, 26 papers were selected for analysis. The following information was extracted from each publication (for the complete dataset see Table A1 in the Appendix section):

- the objective of the study
- the experimental flow (length of exposure to cisplatin, used concentration of cisplatin, conditions of treatment with compound/molecule, the sample size[2])
- mechanism of action of compound/molecule used
- the adverse effects of the compound/molecule
- the outcome measurements

The extracted information was analyzed and the information was summarized and presented in the Results section.

Results

The focus of selected literature

The present review has identified research articles, in which a lateral line of zebrafish was used to study cisplatin-induced hearing loss and otoprotection. The general goals of all the papers were: (i) to identify molecules and compounds protecting the sensory hair cells from cisplatin toxicity (fourteen papers); (i) to develop new knowledge about the mechanisms mediating cisplatin-induced hair cell damage (five papers); (ii) to screen the ototoxicity of various drugs (two papers); (iii) to investigate their synergistically ototoxic effect on hair cells (three articles) and to develop behavioral methods dedicated to zebrafish (two papers).

Zebrafish culture conditions

In all studies, zebrafish embryos were kept at 28.5°C on a 14 h light/10 h dark cycle. The embryos were maintained in Petri dishes in embryo media (EM). Two types of EM were used. The first EM was composed of 15.0 mM NaCl, 0.5 mM KCl, 1.0 mM $MgSO_4$, 0.12 mM KH_2PO_4, 0.074 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 0.5 mM $NaHCO_3$ in distilled water.[17–28] The second EM was compsed of 14.9 mM NaCl, 0.503 mM KCl, 0.994 mM $MgSO_4$, 0.150 mM KH_2PO_4, 42 mM Na_2HPO_4, 0.986 mM $CaCl_2$, 0.714 mM $NaHCO_3$ in distilled water.[12,29–31,32,33] In addition, the embryos were also kept in E3 embryo media consisting of 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 $MgSO_4$ or in a standard bath solution containing: 120 mM NaCl,

2 mM KCl, 10 mM HEPES (*2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid*), 2 mM CaCl2 and 0.7 mM NaH2PO4 adjusted to pH 7.2.[21,34] For the anesthesia, MS-222 (*3-aminobenzoic acid ethyl ester, methanesulfonate salt*) was used. Various sample size was used in the studies, ranging from 3 to 30 zebrafish per experimental group.

Experimental protocols

Based on the literature, cisplatin at low concentrations (50–100 μm) causes the death of lateral line hair cells in zebrafish larvae after 24 h, whereas cisplatin at higher concentrations (250–500 μm) is toxic after 6 h.[11,12] In the selected articles, the zebrafish were exposed to cisplatin for either 24 h (six studies), 6 h (six studies), 4 h (seven studies), and 16 h (one study). In one article, two exposure times were used (12 and 24 h). In the research dedicated to adult zebrafish, the incubation time used was 45 min and 24 h. The results were examined after one cisplatin injection (four articles). In one article, there was no information on that topic.

Various cisplatin concentrations and different exposure times were applied in the studies. Thirteen studies used cisplatin in a concentration lower than 200 μM. Three studies applied cisplatin in a concentration ranging from 200 to 800 μM, whereas seven studies used cisplatin at concentrations ranging between 0.8 and 1 mM (Table 1). In studies focusing at the inner ear of adult zebrafish, 25 mg/kg cisplatin was injected. Two articles provided no information about the concentrations used. The data are summarized in Table 1.

Based on the protocols used, three main experimental designs were identified. In the first design, simultaneous incubation with tested compounds (apocynin, berbamine, edaravone, quercetin, ORC-13661, mdivi-1, NAC, CYM-5478) and cisplatin was used.[20,21,23–25,30] The second experimental strategy used preconditioning with an otoprotective substance (quinoxaline, Z-LLF-CHO, benzamil, quinine, leupeptin, 3-MA, D-methionine, rapamycin, Sal B, SRT1720 and FUT-175) followed by simultaneous incubation with cisplatin.[12,19,31,34,38] The last experimental strategy consisted of preconditioning with otoprotective medication (dexmedetomidine, KR-22335, CHCP1, CHCP2, ATX) and later a sole exposure to cisplatin.[17,26,27,40] In one experimental protocol using adult zebrafish, a single microinjection of cisplatin and drug was applied, whereas when cell cultures were used, the experimental procedure included incubation with cisplatin and then co-incubation with the otoprotective substances.[33,35,36]

In the studies dedicated to apocynin, L-Serine, CHCP1, Rapamycin, Sal B, KR-22335, ORC-13661, ATX, NAC, CYM-5478, and curcuminoids zebrafish served as a second model in addition to mice, guinea pig, cancer cell lines and HEI-OC1 mouse cell line derived from the organ of Corti.[17,20,27,28,30,35,38–40]

The details regarding the experimental design for otoprotective screening are summarized in Table 2.

Berbamine, quinine, and leupeptin offered protection against high concentrations of cisplatin (250–500 μM), whereas ORC-13661, benzamil, L-Serine and mdivi-1 protected against cisplatin used in lower concentration (0–200 μM).[12,19,21,25,30,31,35] Dexmedetomidine, quercetin, and edaravone protected against 1000 μM cisplatin.[23,24,26] FUT-175 (500–1000 μM), quinoxaline (50–400 μM), D-methionine (250–1000 μM), and KR-22335 offered relatively broad protection whereas CHCP2, Sal B, and ATX-LPN exhibited a narrow protection profile (<50 and <60 μM), respectively. Rapamycin and SRT1720 protected the lateral line of larval zebrafish against 12 h exposure to 600 μM cisplatin.[17,28,31,34,38,40] The proteasome inhibitor (Z-LLF-CHO) protected from the exposure to 750 μM cisplatin but had no effect when the lower concentration of cisplatin was used.[31] The curcuminoids offered protection against a single cisplatin injection (25 mg/kg).[36]

Five anti-cancer drugs (sunitinib, raloxifene, dactinomycin, carmustine, and exemestane) were identified as ototoxic substances by using zebrafish larvae.[22] Moreover, drugs such as doxorubicin, vincristine and vinorelbine were shown to have synergistic ototoxic effects.[22] Interestingly, carboplatin caused no ototoxicity in the zebrafish, possibly due to differences in the genome and proteome between mammals and fish, which affects the targeting ability of carboplatin in fish.[22]

Mechanism of otoprotective action of studied compounds and molecules

This review has identified three general strategies used to protect the sensory hair cells from cisplatin-induced damage (see Figure 2).

Table 1. Concentration and duration of exposure to cisplatin (CIS) based on the extracted data.

Article	Cisplatin concentration	Duration of exposure to cisplatin	Experimental flow
Vlasits et al.[12]	0–100 μM	24 h	Pre-treatment for 1 h, then co-exposure for 24 h
Todd et al.[5]	0–1000 μM	4 h	CIS exposure for 4 h
Monroe et al.[35]	100 μM	45 min	Co-exposure for 45 min
Hong et al.[24]	1000 μM	4 h	Co-exposure for 4 h
Lee et al.[23]	1000 μM	4 h	Co-exposure for 4 h
Choi et al.[42]	1000 μM	6 h	Co-exposure for 6 h
Min et al.[26]	1000 μM	6 h	Pre-treatment for 150 min, then CIS exposure for 6 h
Niihori et al.[6]	1000 μM	4 h	Pre-treatment for 12 h, then co-exposure for 4 h
Monroe et al.[33]	100–500 μM	45 min	CIS exposure for 45 min,regeneration for 3 h, then co-exposure for 15 h (cell culture)
Coffin et al.[31]	250–1000 μM	6 h	Pre-treatment for 1 h, then co-exposure for 6 h
Uribe et al.[18]	250–1500 μM	4 h	Co-exposure for 4 h
Kitcher et al.[30]	25–200 μM	24 h	Co-exposure for 24 h
Monroe et al.[36]	25 mg/kg	Single microinjection	CIS microinjection, 24 after drug microinjection
Mackenzie and Raible[29]	50 μM	24 h	Co-exposure for 24 h
Shin et al.[37]	50 μM	24 h	Pre-treatment for 1 h, then CIS exposure for 24 h
Thomas et al.[27]	50 μM	24 h	Pre-treatment for 1 h, then CIS exposure for 24 h
Zheng et al.[38]	50 μM	24 h	Pre-treatment for 2 h, then co-exposure for 24 h
Hirose et al.[22]	50 μM	6 h	Co-exposure for 6 h
Kruger et al.[25]	500 μM	6 h	Co-exposure for 6 h
Vargo et al.[21]	50–200 μM	16 h	Co-exposure for 16 h
Thomas et al.[19]	50–500 μM	6 and 24 h	Pre-treatment for 1 h, then co-exposure for 6 h and 24 h
Rocha-Sanchez et al.[34]	50–800 μM	6 h	Pre-treatment for 2 h, then co-exposure for 6 h
Gu et al.[39]	60 μM	24 h	Pre-treatment for 4 h, then CIS exposure for 24 h
Pang et al.[28]	600 μM	12 and 24 h	Pre-treatment for 1 h, then co-exposure for 12 h and 24 h
Wang et al.[40]	Data not available	24 h	Co-exposure for 24 h

The physiological production of reactive oxygen species is essential for cellular metabolism; however, overproduction or accumulation of ROS can lead to apoptosis. The first type of otoprotective strategy aims at the reduction of overproduction and/or accumulation of ROS to restore cellular homeostasis leading to cell survival. In agreement with the above notion, the otoprotective action of dexmedetomidine, edaravone, KR-22335, apocynin, ATX-LPN, quercetin, and the CHCP1 and CHCP2 molecules were related to their ability to decrease ROS production.[17,20,23,26] Similarly, the EF-24 was suggested to prevent intracellular ROS formation via inhibition of NF-kB-induced signaling and suppressed expression of oncogenic miRNAs, including miR-21.[33] Also dimethyl sulfoxide (DMSO) used at low concentrations is a known scavenger of the hydroxyl radicals.[41]

Apoptosis is a programmed cell death initiated via the intrinsic or extrinsic pathway. The intrinsic pathway can be started by intracellular processes, such as damage to DNA or overproduction of ROS, both known to be induced by cisplatin. In contrast, the extrinsic pathway can be activated by extracellular ligands binding the transmembrane death receptors. At the point of initiation and execution of apoptosis, several proteolytic proteins from the family of caspases and sometimes from the family of calpains may be activated. Targeting the apoptotic or the autophagy pathways by otoprotective substances and compounds may lead to cell survival and is the second type of otoprotective strategy.[42,43] Studies of the inner ear of adult zebrafish have suggested that synthetic curcumin analogs (CLEFMA or EF-24) protect the auditory system against cisplatin-induced damage by inhibition of the apoptotic pathway. Following 48 h exposure to cisplatin, curcuminoids administration induced significant recovery of ABR thresholds (0.1–3 kHz), when compared to cisplatin only.[36] Also, the serin protease inhibitor FUT-175 might function as an apoptosis blocker and protect hair cells from death *via* interaction with the intrinsic and extrinsic apoptotic pathways.[31] Leupeptin, inhibits serine and cysteine proteases—plasmin, trypsin, papain, calpain, and cathepsin B, of which calpain

Table 2. The experimental conditions of otoprotective experiments.

Article	Name of substance	Functional target	The optimal concentration of otoptotective substance	Cisplatin concentration	Experimental flow
Monroe et al.[36]	CLEFMA and EF24	Oxidative stress	5 mg/kg	25 mg/kg	CIS microinjection, 24 h after the compound microinjection
Monroe et al.[35]	L-Serine	Oxidative stress	100 μM	100 μM	Co-exposure for 45 min
Hong et al.[24]	Edaravone	Oxidative stress	750 μM	1000 μM	Co-exposure for 4 h
Lee et al.[23]	Quercetin	Oxidative stress	100 μM	1000 μM	Co-exposure for 4 h
Choi et al.[42]	Apocynin	Oxidative stress	125–250 μM	1000 μM	Co-exposure for 6 h
Kruger et al.[25]	Berbamine	MET-channel	1 and 10 μM	500 μM	Co-exposure for 6 h
Vargo et al.[21]	Mdivi-1	MET-channel	3–7 μM	50-100 μM	Co-exposure for 16 h
Kitcher et al.[30]	ORC-13661	MET-channel	2.2 μM	200 μM	Co-exposure for 24 h
Wang et al.[39]	NAC	Oxidative stress	Data not available	1000 μM	Co-exposure for 24 h
Wang et al.[39]	CYM-5478	Oxidative stress	Data not available	20 μM	Co-exposure for 24 h
Shin et al.[37]	KR-22335	Oxidative stress	1, 10, 100 μg/mL	50 μM	Pre-treatment for 60 min, then CIS exposure for 24 h
Thomas et al.[27]	CHCP2	Oxidative stress	100 μM	50 μM	Pre-treatment for 60 min, then CIS exposure for 24 h
Pang et al.[28]	Rapamycin	Autophagy	10 μM	600 μM	Pre-treatment for 60 min, then co-exposure for 12 h
Pang et al.[28]	SRT1720	Autophagy	5 μM	600 μM	Pre-treatment for 60 min, then co-exposure for 12 h
Vlasits etal.[12]	Benzamil	MET-channel	50 μM	0–100 μM	Pre-treatment for 60 min, then co-exposure for 24 h
Coffin et al.[31]	Leupeptin	Calpains	500 μM	250 μM	Pre-treatment for 60 min, then co-exposure for 6 h
Coffin et al.[31]	Z-LLF-CHO	Protease	25 μM	750 μM	Pre-treatment for 60 min, then co-exposure for 6 h
Coffin et al.[31]	FUT-175	Proteasome	10 μM	500–1000 μM	Pre-treatment for 60 min, then co-exposure for 6 h
Coffin et al.[31]	D-methionine	Oxidative stress	5 mM	250–1000 μM	Pre-treatment for 60 min, then co-exposure for 6 h
Coffin et al.[31]	3-MA	Autophagy	5 mM	250, 750 μM	Pre-treatment for 60 min, then co-exposure for 6 h
Thomas et al.[19]	Quinine	MET-channel	100 μM	250–500 μM	Pre-treatment for 60 min, then co-exposure for 6 h
Zheng et al.[38]	Sal B	Oxidative stress	40 μM	0–50 μM	Pre-treatment for 120 min, then co-exposure for 24 h
Rocha-Sanchez et al.[34]	Quinoxaline	Oxidative stress	300 μM	50–400 μM	Pre-treatment for 120 min, then co-exposure for 6 h
Min et al.[26]	Dexmedetomidine	Oxidative stress	0.1, 1, 10 μM	1000 μM	Pre-treatment for 150 min, then CIS exposure for 6 h
Gu et al.[40]	ATX-LPN	Oxidative stress	50 μg/mL	60 μM	Pre-treatment for 240 min, then CIS exposure for 24 h

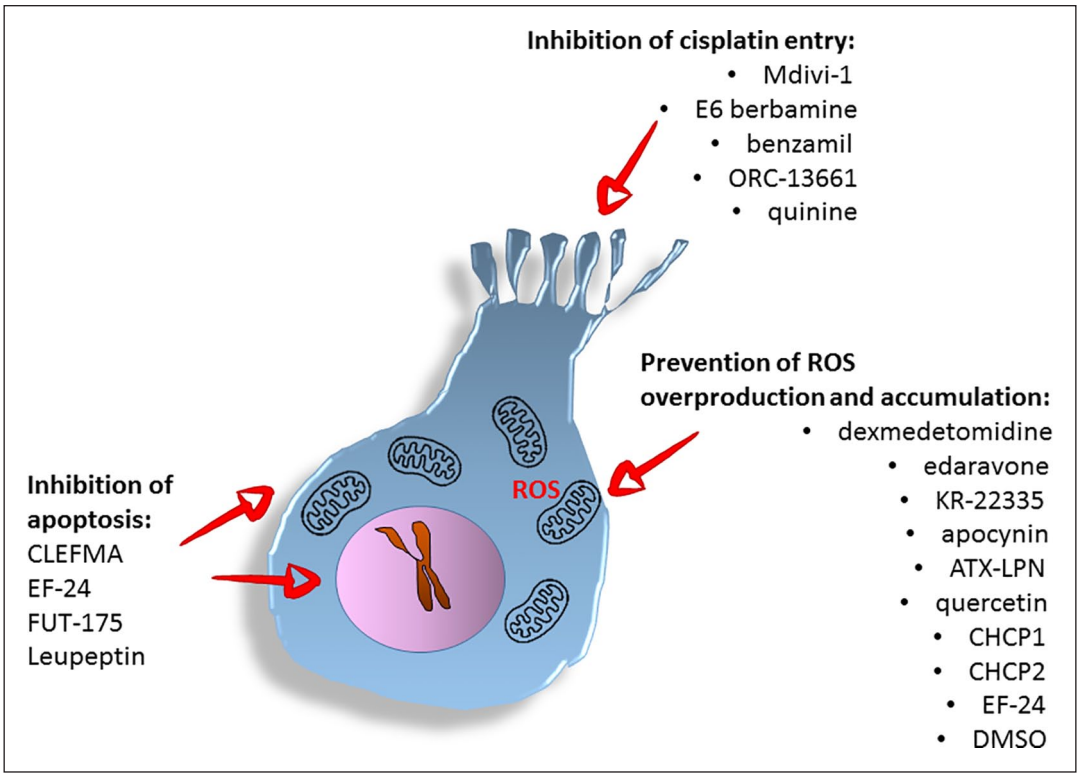

Figure 2. Summary of anti-ototoxic strategies and respective compounds.

and cathepsin were implicated to be involved in apoptotic processes.[44]

The therapeutic impact of quinoxaline (Qx) against cisplatin was attributed to the prevention of apoptosis of the sensory hair cells in a still indeterminate way.[34] The cellular process of autophagy, in which the damaged mitochondria are being eliminated from the cells, thus, promoting cell survival, can counteract apoptosis.[45] In agreement with that, the experiments demonstrated that the exposure of larval zebrafish to autophagy modulators (3-MA and rapamycin) prevented the cisplatin-induced damage to the lateral line.[28,31]

The third otoprotective strategy involves blocking the entry of toxic substances into the inner ear and, in particular, into hair cells.[31,46] Lateral line hair cells share mechanisms of mechanotransduction (MET) with hair cells of the inner ear.[19] Data in the literature suggest that blocking MET channel prevents the intracellular accumulation of cisplatin.[19] Currently, it is unclear if cisplatin enters the lateral line of zebrafish directly through the MET channels. However, studies using blockers of MET channels (Mdivi-1, E6 berbamine, benzamil and ORC-13661, quinine) in larval zebrafish have confirmed that blocking of MET channels protects from the cisplatin-induced hair cell death.[12,19,21,25] Lastly, blockade of mechanotransduction using quinine protected from the cisplatin-induced hair cell loss. However, quinine is a well-known ototoxin; therefore, its medical usefulness as an otoprotector might be negligible.[19,47]

The adverse effects of the tested compounds/molecule

The adverse effects of the compounds and molecules used as otoprotectors were identified during data extraction. The adverse effect was found to be time and dose-dependent. Mdivi-1 in doses higher than 10 µM was toxic to zebrafish. CHCP1 started to be lethal, for the zebrafish larvae, in concentrations above 100 µM.[21] On the one hand, Z-LLF-CHO presented otoprotective properties; however, continuous exposure to Z-LLF-CHO was toxic to the hair cells.[31] A similar dependence was ecountered with DMSO at a concentration of 0.01%, which was toxic to zebrafish, as opposed to lower concentration which could induce otoprotection.[48] The DMSO concentration of 0.5% or higher were shown to be toxic to the auditory hair cells in the rat cochlear explant cultures.[49] In combination

with cisplatin, DMSO has induced more extensive hair cell death than cisplatin alone.[18] The protective effect of quinoxaline depends on the incubation protocol. The otoprotection D-methionine was limited to incomplete hair cell survival seen in all cases.[31] Incubation with flubendazole during recovery after cisplatin exposition blocked producing the new hair cells in zebrafish.[50] The adverse effects of other substances were not reported.

Outcome measurements

In the majority of the reviewed studies, the assessment of the otoprotective and ototoxic effects involved sensory hair cell counting (eighteen articles).[12,17,18,20–22,24,25,27,28,30,31,32–34,39,40] An additional method, evaluating the hair cell function, was the uptake of FM1-43 dye (four articles) and the recording of microphonic potentials (two articles).[19,21,25,34,43] In six articles, the TUNEL assay was applied, detecting single-stranded brakes in the chromosomal DNA (a feature of apoptosis).[20,23,24,34,37,38] The proliferation assay was used in two articles, whereas the fluorescent platinum analog (Rho-Pt) uptake assay was used in one article.[27,29,34] In two studies, anatomic changes were correlated with behavioral modification observed in the zebrafish with the help of rheotaxis.[6,32] In another two studies, in which the inner ear cell of zebrafish was cultured, spectrophotometry was used.[33,35] Lastly, one publication assessed the hearing abilities of adult zebrafish by measuring auditory brainstem responses (ABR).[36]

Discussion

This review aimed to assess the usefulness of the lateral line in zebrafish for studying cisplatin-induced ototoxicity and otoprotection. Twenty-two studies published between January 2009 and May 2020 dedicated to the zebrafish larvae and four dedicated to the studies of the inner ear in adult zebrafish met the inclusion criteria. All articles have confirmed the usefulness of the lateral line in the high-throughput screening of otoprotective substances. These studies employed anatomical and behavioral assays to measure the experimental outcome.

In the inner ear, cisplatin accumulates predominantly in the stria vascularis. However, the most significant damage induced by cisplatin is seen in the sensory hair cells, the supporting cells in the mammalian vestibular system (utricle), and the regenerative potential of the utricle.[14,51,52] Cisplatin induces cytotoxicity by binding to the nuclear DNA, leading to apoptosis, particularly in the proliferating cells.[53] In addition to that, cisplatin can mediate the activation of NADPH oxidase 3 (NOX3), which catalyzes the production of superoxide, representing reactive oxygen species (ROS). Overproduction of ROS can activate the signal transducer and activator of transcription 1 (STAT1), inducing the inflammation.[46] Previous studies have shown that ROS might induce autophagy, which, depending on the stimulation context, can promote either cell survival or lead to cell death.[54,55]

The critical step responsible for the ototoxic properties of cisplatin is its transport inside the sensory cell. Several studies have reported that mechanotransducer (MET) channels mediate the entry of cisplatin into cochlear hair cells, but it remains unclear whether MET channels are blocked during that process.[30,46,53] A study using larval zebrafish confirmed that cisplatin-induced damage to the hair cells relies on functional MET channels.[19] Furthermore, the effect of platinum (II) complexes on adult zebrafish auditory system suggested that cisplatin, and to a limited extent phenanthriplatin, can induce hair cells loss in particular regions of the saccule but not the utricle,[56] suggesting either various regional susceptibility to cisplatin or its distinct diffusion or transport pattern.

Studies using larvae zebrafish included in the present review confirmed the otoprotective properties of astaxanthin (ASX), N-acetylcysteine (NAC), rapamycin, aalvianolic acid B, SRT1720, E6 berbamine, quercetin, dexamethasome, dexmedetomidine, edaravone, quinine, dimethyl sulfoxide (DMSO), FUT-175, benzamil, apocynin, and flubendazole. What is more, the clinical adverse effects of the above molecules are already known, as ASX, NAC, rapamycin, dexamethasone, quinine, dexmedetomidine, DMSO, edaravone, flubendazole are used in clinical trials or practice (see Table 3). Interestingly, the screening of new molecules by the zebrafish model, identified new otoprotective compounds such as CHCP1, CHCP2, apocynin, quinoxaline, ORC-13661, SRT1720, CYM-5478, mdivi-1, KR-22335, leupeptin, and 3-MA. Although no information is yet available on the impact of these compounds on cisplatin-efficiency, their small size makes a local delivery

possible, which could be a solution to avoid the risk of antitumor interference and other adverse effects.

The otoprotective effect of various substances observed in zebrafish was also demonstrated in other animal models and humans. The administration of dexamethasone delivered by intratympanic injection in cancer patients provided narrow protection against hearing-loss (at 6 kHz).[3] Lack of otoprotection was observed in patients who have received cisplatin chemotherapy and an injection of poloxamer hydrogel containing dexamethasone (OTO-104).[99] Currently, the OTO-104 is tested in patients with unilateral Meniere's Disease (administrated by a single intratympanic injection).[100] Animal studies with guinea pigs demonstrated an otoprotective effect of dexamethasone only in co-administration with curcumin, whereas when both substances were administrated alone provided no protection in the zebrafish.[26,36,101] To consider that data in the literature show that dexamethasone reduces the cisplatin efficiency.[65]

N-Acetylcysteine (NAC) which was found protective in zebrafish, presented conflicting results in humans. One study found that a local delivery of NAC provided an otoprotection at 8 kHz (using the patient's opposite ear as a control);[70] another study by Yoo et al, reported no differences between cancer patients treated with NAC and an untreated group.[102] There was no protective effect of an oral low-dose NAC in patients with head and neck cancer from cisplatin-induced toxicities and oxidative stress.[103] Currently, there is an IV phase clinical trial of the effectiveness of intratympanic administration of N-acetylcysteine in patients treated with cisplatin.[104]

Studies in zebrafish confirmed the otoprotective effect of D-methionine observed in guinea pigs. Guinea pigs, after a local application of D-methionine in the round window, presented improved otoacoustic emissions.[105] Multiple studies have demonstrated the D-methionine protection against cisplatin, amikacin and on permanent noise-induced hearing loss.[106] Nevertheless, there is no information about the effect of D-methionine on cancer cells. Currently, the efficacy of L-serine, astaxanthin (ATX) and rapamycin are assessed *in clinical trials* for treating Alzheimer's disease, glucose intolerance and *amyotrophic lateral sclerosis (ALS), which will help us understand their adverse effects.*[107–109]

Similarities observed in the results obtained from different animal models and humans, support the idea of using of zebrafish for preclinical drug-screening. Nevertheless, not all substances identified by the zebrafish are acceptable for use in humans. The differences between results in otoprotective studies in humans and animals are caused by differences in the drug administration protocol, cohort size and cancer types.[110]

The majority of studies used anatomical or histological/immunohistological assays considered a gold standard in the ototoxicity research to measure the experimental outcome. Few studies employed specific, zebrafish-related behavioral methods such as rheotaxis. The rheotaxis uses the physiological principle of fish facing the oncoming current of water, which can be already observed in zebrafish larvae because the lateral line is entirely sensitive to the environment from 5 dpf.[111] There is a dose-dependent relationship between cisplatin exposure, progressive hair cell damage, and reduced fish swimming behavior. Moreover, in response to otoprotective substances such as dexamethasone (5 μM+ cisplatin 1000 μM), the rheotaxis of zebrafish improved significantly.[6] These results indicate that detecting changes in the swimming behavior could serve as a biomarker for the functionality of hair cells of fish. The automated swimming apparatus provides quick testing of large numbers of zebrafish and opens a new field in the ototoxic studies.[6,32] Nevertheless, a few model limitations must be considered. Firstly, not only the lateral line but also the visual system are essential for rheotaxis.[112] Secondly, higher concentrations of cisplatin may affect other systems of zebrafish (e.g., neurotransmitters or motor neurons), changing their swimming behavior.[6]

While collecting data for the present review, we have identified four articles dedicated to adult zebrafish inner ear (⩾6 mpf) and cisplatin-induced ototoxicity that were published between 2009 and 2020. Two of the manuscripts were studying the curcuminoids-dependent otoprotection against cisplatin.[33,36] The third article focused on the effect of the platinum (II) complex on ABR hearing thresholds and found that the exposure to platinum (II) complex resulted in decreased hearing thresholds similarly as in response to cisplatin.[56] The aim of the fourth article was to study whether L-serine might impact on the reduction of cisplatin-mediated ROS generation in vestibular tissue.[35]

The zebrafish larvae model offers two significant advantages: (i) it can be used during a screening of

Table 3. Candidate molecules and compounds for the protection against cisplatin induced-hearing loss.

Molecule or compound	IUPAC name	Natural occurrence	Direct effect on cancer cells	Modulation of cisplatin anti-cancer cytotoxicity	Known adverse effects in humans	Use in human trials
3-MA	**3**-Methylpentane	A structural isomer of hexane	No data available	No data available	Irritation, headache, drowsiness, dizziness, loss of coordination, convulsions, and coma[57]	Technically possible
Apocynin	1-(4-hydroxy-3-methoxyphenyl)ethanone	Apocynin is a natural polyphenolic compound isolated from various plants such as *Apocynum cannabinum, Picrorhiza kurroa*	Apocynin inhibits the NF-kB activation in androgen-independent rat prostate cancer cell lines[58]	No data available	Irritating to eyes, respiratory system and skin[59]	No data available
Astaxanthin (ATX)	3,3'-Dihydroxy-beta,beta-carotene-4,4'-dione	Astaxanthin is a keto-carotenoid in the terpenes class of chemical compounds. It is classified as a xanthophyll but it is a carotenoid with no vitamin A activity. It is found in the majority of aquatic organisms with red pigment.	Enhances CTL activity and IFN-gamma production against Meth-A tumor cells in parallel to suppression of tumor growth[60]	No data available	Doses of up to 50 mg have been tolerated. An upper toxicity limit is not known[61]	Technically possible
Benzamil	3,5-diamino-N-(N'-benzylcarbamimidoyl)-6-chloropyrazine-2-carboxamide	Synthetic molecule	No data available	Amiloride (10, 30, and 100 μmol/L) concentration-dependently potentiated erlotinib-induced inhibition of cell proliferation and colony formation in the four pancreatic cancer cell lines[62]	Irritating to eyes, respiratory system and skin[63]	Technically possible
CHCP1	Not available	Not available	Not available	Not available	Not available	No data available
CHCP2	Not available	Not available	Not available	Not available	Not available	No data available
CLEFMA	(Z)-4-[(3E,5E)-3,5-bis[(2-chlorophenyl) methylidene]-4-oxopiperidin-1-yl]-4-oxobut-2-enoic acid	Synthetic molecule	IC_{50} for the human lung carcinoma A549 cell line measured by MTT test - 13.82 μM (24 h) and 16.05 (48 h)	Decreases the cisplatin-induced ROS production, decreases the motility of cancer cells in the presence of cisplatin[36]	No data available	No data available
CYM-5478	2-[[4-[5-(3,4-diethoxyphenyl)-1,2,4-oxadiazol-3-yl]-2,3-dihydro-1H-inden-1-yl]amino]ethanol	No data available	No data available	No data available	No data available	No data available
Dexamethasone	(8S,9R,10S,11S,13S,14S,16R,17R)-9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-3-one	Dexamethasone is a synthetic adrenal corticosteroid	Induction of apoptosis and enhancement chemosensitivity of GRα-rich colon cancer cell lines[64]	Dexamethasone reduces cisplatin efficiency in different human NSCLC cell lines in a p53-dependent manner[65]	Vertigo, acne, insomnia, increased appetite, irritability, muscle weakness, impaired wound healing, amnesia increased blood sugar levels[66]	Technically possible
Dexmedetomidine	5-[(1S)-1-(2,3-dimethylphenyl)ethyl]-1H-imidazole	Dexmedetomidine is an imidazole derivate and active d-isomer of medetomidine	Dexmedetomidine promots cell proliferation, migration and upregulats antiapoptotic protein in human lung carcinoma and neuroglioma cell lines[67]	no data available	low or high blood pressure (hypotension or hypertension), slow heart rate (bradycardia), nausea, dry mouth, irregular heartbeat, fever, vomiting, low blood plasma[68]	technically possible

(Continued)

Table 3. (Continued)

Molecule or compound	IUPAC name	Natural occurrence	Direct effect on cancer cells	Modulation of cisplatin anti-cancer cytotoxicity	Known adverse effects in humans	Use in human trials
Dimethyl sulfoxide (DMSO)	Methylsulfinylmethane	Synthetic molecule	DMSO inhibits the tumor volume growth in breast cancer bearing mice (except for 0.25 mg/g) in a time and dose-dependent way.[37]	DMSO did not have any effect on cisplatin's cytoxicity on human cervical (KB-3-1) or colorectal (DLD-1) carcinoma cells[69]	Garlic-like taste (a few minutes after instillation), odor on breath [70]	Technically possible
D-methionine	(2R)-2-amino-4-methylsulfanylbutanoic acid	D-methionine is an optically active form of methionine having D-configuration. It is a methionine and a D-alpha-amino acid	No data available	No data available	No data available	Technically possible
E6 Berbamine	(20,21,25-trimethoxy-15,30-dimethyl-7,23-dioxa-15,30-diazaheptacyclo[22.6.2.23,6.18,12.114,18.027,31.022,33] hexatriaconta-3(36),4,6(35),8,10,12(34),18,20,22(33),24,26,31-dodecaen-9-yl) 4-nitrobenzoate	Berbamine is a natural compound derived from the Berberis amurensis plant, E6 Berbamine is a synthetic molecule	Induction of apoptosis in breast cancer and lung cancer cell lines[71]	No data available	Jaundice, stomach upset, lethargy, nose bleed, skin and eye irritation, kidney irritation[72]	Technically possible
Edaravone	5-methyl-2-phenyl-4H-pyrazol-3-one	Not available	Lack of cytostatic effect in colon carcinoma cells[73]	No data available	Bruising, gait disturbance, headache,skin inflammation or rash, eczema, respiratory disorder,oxygen deficiency[74]	Technically possible
EF24	(3E,5E)-3,5-bis[(2-fluorophenyl)methylidene] piperidin-4-one	Synthetic molecule	Cytostatic effect[75]	Decreases the cisplatin-induced ROS production, decreases the motility of cancer cells in the presence of cisplatin[36]	No data available	No data available
Flubendazole (Flu, microtubule assembly blocker)	Methyl N-[6-(4-fluorobenzoyl)-1H-benzimidazol-2-yl]carbamate	Flubendazole is a member of the class of mebendazole. The benzoyl group is replaced by a p-fluorobenzoyl group	Flubendazole inhibited breast cancer cells proliferation in dose- and time-dependent manner[50]	No data available	Abdominal pain, headache, dizziness, diarrhoea[76]	Technically possible
FUT-175	(6-carbamimidoylnaphthalen-2-yl) 4-(diaminomethylideneamino) benzoate;methanesulfonic acid	Nafamostat Mesylate (MN) is the mesylate salt form of nafamostat	NM significantly inhibits proliferation, migration, and invasion in MDA-MB231 triple-negative breast cancer (TNBC) cells[77]	No data available	Nausea, vomiting, itching and eruption[78]	Technically possible

(Continued)

Table 3. (Continued)

Molecule or compound	IUPAC name	Natural occurrence	Direct effect on cancer cells	Modulation of cisplatin anti-cancer cytotoxicity	Known adverse effects in humans	Use in human trials
KR-22335	3-Amino-3-(4-fluoro-phenyl)-1H-quinoline-2,4-dione	Synthetic molecule	No data available	KR-22332 does not appear to interfere with the antitumor effect of chemotherapeutic[37]	No data available	No data available
Leupeptin	N-Acetyl-L-leucyl-L-leucyl-L-argininal hemisulfate salt	Organic compound produced by actinomycetes	No data vailable	No data availabe	No data available	Technically possible
L-serine	(S)-2-Amino-3-hydroxypropanoic acid H-Ser-OH;	Serine is a nonessential amino acid derived from glycine. L-serine is the L-enantiomer of serine.	No data available	L-serine inhibits the antitumor effect of cisplatin in human gastric cancer cell lines SGC7901, BGC823, and MGC803 in a dose-dependent manner[79]	Tiredness, anxiety, chronic fatigue, neurotoxicity, depression[80]	Technically possible
Mdivi-1	3-(2,4-dichloro-5-methoxyphenyl)-2-sulfanylidene-1H-quinazolin-4-one	Synthetic molecule	No data available	Synergistic action with cisplatin[81]	No data available	No data available
NAC	N-Acetyl-L-cysteine ((2R)-2-acetamido-3-sulfanylpropanoic acid)	NAC is an essentially prodrug that is converted to cysteine (in the intestine by the enzyme aminoacylase 1) and absorbed in the intestine into the blood stream. Cysteine is a key constituent to glutathione and hence administration of acetylcysteine replenishes glutathione stores.	Growth inhibition of several cancers[82]	No data available	Unlikely cause of clinically apparent liver injury[83]	Technically possible
ORC-13661	(1R,8S)-4-[(4-chlorophenyl)carbamoylamino]-11-methyl-5-thia-11-azatricyclo[6.2.1.02,6]undeca-2(6),3-diene-3-carboxamide	No data available	No data available	No data available	No data available	No data available
Quercetin	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one	Quercetin is a flavonoid widely distributed in many plants, vegetables and fruits	Growth inhibition in the human breast carcinoma cell line[58]	Synergistic action with cisplatin against human oral squamous cell carcinoma (OSCC) (cell lines Tca-8113 and SCC-15)[84]	Headache (oral use), numbness and tingling (oral use), shortness of breath (intravenous use), nausea and vomiting (intravenous use), kidney damage (intravenous use greater than 945 mg/m^2)[85]	Technically possible

(Continued)

Table 3. (Continued)

Molecule or compound	IUPAC name	Natural occurrence	Direct effect on cancer cells	Modulation of cisplatin anti-cancer cytotoxicity	Known adverse effects in humans	Use in human trials
Quinine	(R)-[(2S,4S,5R)-5-ethenyl-1-azabicyclo[2.2.2]octan-2-yl]-(6-methoxyquinolin-4-yl)methanol	Quinine is a quinidine alkaloid isolated from the bark of the cinchona tree	Quinine possess cytotoxic effect on laryngeal cancer cells (147.58 μM/mL for 24 h and 123.74 μM/mL for 48 h)[86]	No data available	Headache, ringing in the ears, trouble seeing, and sweating, deafness, low blood platelets, an irregular heartbeat, during pregnancy is harm to the baby[87]	Technically possible
Quinoxaline	Quinoxaline	Synthetic molecule	Cytostatic effect[88]	No data available	No data available	No data available
Rapamycin	(1R,9S,12S,15R,16E,18R,19R,21R,23S,24Z,26E,28E,30S,32S,35R)-1,18-dihydroxy-12-[(2R)-1-[(1S,3R,4R)-4-hydroxy-3-methoxycyclohexyl]propan-2-yl]-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-azatricyclo[30.3.1.04,9]hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentone	Isolated from Streptomyces hygroscopicus found in an Easter Island soil sample	Used for therapy of carcinoma[89] leukemia (B-CLL)[90] melanoma[91]	Synergistic action with cisplatin against various types of cancer[92,93]	Stomatitis, behavioral disturbance, rash, pyrexia, pneumonia, gastroenteritis, aggression, agitation, amenorrhea, hypercholesterolemia, elevated partial thromboplastin time, neutropenia, infectiorapa[94]	Technically possible
Salvianolic acid B (Sal B)	(2R)-2-[(E)-3-[(2R,3R)-3-[(1R)-1-carboxy-2-(3,4-dihydroxyphenyl)ethoxy]carbonyl-2-(3,4-dihydroxyphenyl)-7-hydroxy-2,3-dihydro-1-benzofuran-4-yl]prop-2-enoyl]oxy-3-(3,4-dihydroxyphenyl)propanoic acid	Sal B is a compound extracted from Salvia miltiorrhiza (Danshen)	Slows the growth of breast cancer cells[95] and and lung cancer cells[96]	No data available	Allergy, dizziness, headache, mild GI symptoms, and reversible thrombocytopenia[97]	Technically possible
SRT1720 (small-molecule activator of the sirtuin subtype SIRT1)	N-[2-[3-(piperazin-1-ylmethyl)imidazo[2,1-b][1,3]thiazol-6-yl]phenyl]quinoxaline-2-carboxamide;2,2,2-trifluoroacetic acid	No data available	Cytostatic effects on various cancer[88]	No data available	Stomatitis, behavioral disturbance, rash, pyrexia, pneumonia, gastroenteritis, aggression, agitation, amenorrhea, hypercholesterolemia, elevated partial thromboplastin time, neutropenia, infection	Technically possible
Z-LLF-CHO	Benzyl N-[(2S)-4-methyl-1-[[(2S)-4-methyl-2-[(1-oxo-3-phenylpropan-2-yl)amino]pentanoyl]amino]-1-oxopentan-2-yl]carbamate	No data available	Z-LLF-CHO induces early tumor regression and a delay in tumor progression in a murine model of Burkitt's lymphoma[98]	No data available	No data available	No data available

novel substances and compounds against cisplatin-induced hair cell damage; (ii) it can promote a better understanding of the mechanisms related to cisplatin-induced hearing loss.[21] However, studies using zebrafish are not free of limitations. The first limitation is the ability of zebrafish hair cells to regenerate through the proliferation of supporting cells, which is not the case for mammalian hair cells. Subsequently, diverse damage protocols appear to induce different pathways leading to hair cell loss.[111] This implies the importance of testing zebrafish over a longer time. The second limitation is the fact that the zebrafish hearing range is low and does not reflect this of humans, whereas cisplatin primarily affects the hair cells in high frequencies.[113] In addition, larvae are sensitive to frequencies up to 1200 Hz, while the inner ear hair cells of the adult zebrafish can detect sound frequencies up to 4000 Hz. The inner ear of zebrafish is sensitive to a broader spectrum of frequencies, whereas the zebrafish lateral line is restricted to detecting low-frequency sounds.[113,114] The third limitation is the lack of a stria vascularis in contrast to the human inner ear; thus, damage through strial mechanism could not be evaluated.[24] The fourth limitation is that the zebrafish shares only 70% homology with the human genome, and therefore, some proteins that in humans will be targeted by the ototoxic drugs may no longer be a good target, or simply are absent in fish. It suggests that zebrafish could be used as a model only for studying acute ototoxicity at low frequencies; however, some findings may still be applicable to humans.[19] The fifth pitfall of the presented studies is the varying sample size. *Because of the character of our review, the sample size was not used as an exclusion criterion.* In large part, the ototoxic studies focused on the zebrafish lateral line. In contrast, it is supposed that the zebrafish's inner ear may display different sensitivity to drugs or different times of response and regeneration.[14] Nonetheless, the zebrafish lateral line is a relatively easy, quick, and inexpensive alternative to the ototoxicity studies in rodents.

The studies using zebrafish larvae demonstrated that cisplatin-induced hair cell loss could be reduced by lowering the levels of ROS, by the apoptosis and by inhibiting the MET channel. However, the effectiveness of substances and compounds tested still has to be proven under other experimental conditions. Importantly, the present review identified significant discrepancies between the protocols used, suggesting a need for the establishment of a consensus method to test the anti-ototoxic properties of compounds in zebrafish. These protocols should implement optimized concentrations of cisplatin and standardized incubation times. A practical suggestion to improve data presentation is using RDI (relative dose intensity), reflecting the ratio of "delivered" to the "planned" dose intensity and can be expressed as a percentage.

Conclusion

Despite a relatively low number of studies in the past 12 years, zebrafish prove to be a useful model for studying ototoxicity, especially during high throughput screening of new ototoxic compounds. However, the present study identified a need for developing a consensus protocol that should be used during future ototoxic studies. It is recommended to develop a standardized range of cisplatin concentration, duration of exposure, and the sequence of exposure concerning tested compounds. The generation of an agreed-upon experimental protocol should be on the priority list of researchers using the zebrafish model to study ototoxicity. This field of science would also benefit from standardization of outcome measures and units used, as well as from precise specification of observed effects and molecules (e.g., instead of using ROS, referring to a precise molecule that is produced, such as peroxide, superoxide, hydroxyl radical, singlet oxygen, or alpha-oxygen). Finally, even though zebrafish does not offer an ideal model for cisplatin-induced hearing loss, the mechanism of the damage to zebrafish hair cells is likely similar to that in humans, which is encouraging for further use of this model in ototoxicity and otoprotection-related studies.

List of abbreviations

3-MA	3-methyladenine
ATX	Astaxanthin
CHCP1	Cisplatin Hair Cell Protectant 1
CHCP2	Cisplatin Hair Cell Protectant 2
CYM-5478	an S1P2 selective agonist
Danio rerio	zebrafish
dpf	days post-fertilization
EM	embryo media
FUT-175	Nafamostat Mesilate
KR-22335	3-Amino-3-(4-fluoro-phenyl)-1H-quinoline-2,4-dione

mdivi-1	Mitochondrial Division Inhibitor 1
MET	mechanotransducer
NAC	N-acetylcysteine
ORC-13661	Oricula Therapeutics' first product
RDI	relative dose intensity
ROS	Reactive oxygen species
SRT1720	a specific SIRT1 activator/ a cell-permeable inhibitor of the mitochondrial SIRT3
Z-LLF-CHO	benzyl N-[(2S)-4-methyl-1-[[(2S)-4-methyl-2-[(1-oxo-3-phenylpropan-2-yl)amino]pentanoyl]amino]-1-oxopentan-2-yl]carbamate

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Supplemental material

Supplemental material for this article is available online.

Notes

1. When in addition to zebrafish, other animals were studied, only the data related to zebrafish were acquired.
2. The sample size was not considered as an exclusion criteria.

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