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# Regulatory mechanisms of miR-96 and miR-184 abnormal expressions on otic vesicle development of zebrafish following exposure to β-diketone antibiotics

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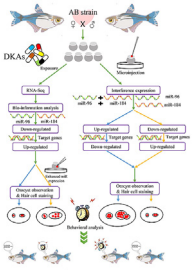
## HIGHLIGHTS

- miR-96 and -184 down-regulation following DKA exposure during embryonic development.
- miR-96 play a critical role in otic vesicle development and formation of hearing.
- miR-96 influences otic vesicle development by affecting hair cell differentiation.
- miR-184 is involved in otic vesicle construction during embryonic development.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Chronic ototoxicity of β-diketone antibiotics (DKAs) to zebrafish (*Danio rerio*) was explored in detail by following abnormal expressions of two hearing-related miRNAs. Dose-dependent down-regulation of miR-96 and miR-184 was observed in otoliths during embryonic-larval development. Continuous DKA exposure to 120-hpf larva decreased sensitivity to acoustic stimulation. Development of otolith was delayed in treatment groups, showing unclear boundaries and vacuolization at 72-hpf, and utricular enlargement as well as decreased saccular volume in 96-hpf or latter larval otoliths. If one miRNA was knocked-down and another over-expressed, only a slight influence on morphological development of the otic vesicle occurred, but knocked-down or over-expressed miRNA both significantly affected zebrafish normal development. Injection of miR-96, miR-184 or both micRNA mimics to yolk sac resulted in marked improvement of otic vesicle phenotype. However, hair cell staining showed that only the injected miR-96 mimic restored hair cell numbers after DKA exposure, demonstrating that miR-96 played an important role in otic vesicle development and formation of hearing, while miR-184 was only involved in otic vesicle construction during embryonic development. These observations advance our understanding

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of hearing loss owing to acute antibiotic exposure and provide theoretical guidance for early intervention and gene therapy for drug-induced diseases.



## 1. Introduction

Fluoroquinolones (FQs) and tetracyclines (TCs) are two important classes of pharmaceutical and personal care products designated as β-diketone antibiotics (DKAs) due to the presence of a β-diketone functional group in their molecular structure. DKAs are widely utilized because of their excellent broad-spectrum antibacterial effect (Qu et al., 2010; Yoon et al., 2010). Frequent usage and continuous emission of DKAs into the environment result in DKAs becoming a "pseudo-persistent" pollutant, in spite of their comparatively short half-lives. As a result, DKAs can lead to metabolic and cumulative toxicity, and cause ecosystem pressure on numerous organisms (Brain et al., 2005; Sarmah et al., 2006). Toxicity of FQs and TCs has been widely reported, including a range of cardiovascular diseases and arthropathy (Mulgaonkar et al., 2012). FQs can significantly inhibit the activity of acetylcholinesterase, leading to potential neurotoxicity (Wang et al., 2009). Additionally, TCs cause impaired organ metabolism and fatty liver formation (Yin et al., 2006), and inhibit the activity of superoxide dismutase in liver causing metabolic disturbance in zebrafish embryos (Wang et al., 2014).

In real-world environments, residual components of antibiotics are very complex and may contribute to joint (i.e., interactive) toxicological effects (Ding et al., 2013; Melvin et al., 2014). Our previous research examined the effects of three representative FQs (enrofloxacin, ofloxacin and ciprofloxacin) and three TCs (chlortetracycline, oxytetracycline and doxycycline) on joint toxicity to various tissues and organs including the immune, reproductive and nervous systems in zebrafish (*Danio rerio*) (Li et al., 2016a). Some FQ family members, such as ciprofloxacin (Etminan et al., 2017; Samarei et al., 2014), are reported to cause ototoxic effects, while some TC family members, such as doxycycline (Chotmongkol et al., 2012) and minocycline (Corbacella et al., 2004) function to restore hearing loss. However, there is a paucity of data regarding chronic joint DKA exposure on hearing impairments, especially studies examining the molecular response to DKA exposure.

Li and coworkers reported abnormal expression of a large number of microRNAs (miRNAs) after DKA exposure based on high throughput RNA sequencing (Li et al., 2016b). The miRNAs are an abundant class of non-coding RNAs that can potentially control dozens of genes. Multiple miRNAs have been shown to collaborate in targeting extensive cellular processes and molecular pathways (Vlachos et al., 2015; Wienholds and Plasterk, 2005b). Numerous studies attributed abnormal miRNA expression and regulation to a variety of diseases, such as cancer (Kaur et al., 2016; Zhao et al., 2017), heart disease (Yang et al., 2017) and brain development (Xu et al., 2017). For example, miR-96 was reported as a hearing loss factor (Lewis et al., 2016; Soldà et al., 2012), and miR-184 showed a high correlation with cancer (Zhou et al., 2017) and nervous system development (Liang et al., 2017; McKiernan et al., 2012). Although our previous studies speculated that miR-96 and miR-184 might have some functional relevance (Li et al., 2016b), no direct evidence or additional reports have examined their contributions to molecular mechanisms regulating hearing functions. Thus, additional research is highly warranted from a public health perspective given that some FQs may cause ototoxicity (Etminan et al., 2017; Samarei et al., 2014) while some related TCs function to restore hearing loss (Chotmongkol et al., 2012; Corbacella et al., 2004). Notably, the underlying molecular mechanisms concerning DKA impacts on human hearing loss have yet to be rigorously investigated.

Based on our previous research (Li et al., 2016b), miR-96 and miR-184 were identified as key factors warranting further study to investigate the role of DKAs on hearing functions. Therefore, this research focused on expression changes of miR-96 and miR-184, their target genes and functional relevance in response to DKA exposure of zebrafish embryos. The effects of abnormal expressions of miR-96 and miR-184 on otic vesicle development were investigated in detail using manual intervention of miRNA expression, behavioral analysis, morphological observations and hair cell staining. The findings of this study further our understanding of deafness diseases due to antibiotic exposure, and also provide theoretical guidance for early intervention and gene therapy for drug-induced diseases.

## 2. Material and methods

### 2.1. Ethics statement

The Institutional Animal Care and Use Committee (IACUC) at Wenzhou Medical University approved our study plan for ethical use of zebrafish (*Danio rerio*). All studies were carried out in strict accordance to IACUC guidelines. Dissection was performed on ice to minimize suffering.

### 2.2. Chemical reagents and exposure protocols

Six DKAs were purchased from Amresco (Solon, OH, USA); CAS number, purity, chemical structure and molecular weight are shown in Table S1 and Fig. S1. Wild type adult zebrafish (AB strain) were purchased from a local supplier and adapted to laboratory conditions with a light/dark, 14 h/10 h cycle in a circulation system with dechlorinated tap water (pH 7.0–7.5) at a constant temperature ($27 \pm 0.5\,^{\circ}C$). Zebrafish maintenance followed Westerfield (1995). Embryos were exposed to a series of DKA concentrations (0, 12.5 and 25 mg $L^{-1}$ (25 mg $L^{-1}$ corresponding to 11.5, 12.6, 11.6, 8.1, 8.1 and 9.0 μM for ofloxacin, ciprofloxacin, enrofloxacin, doxycycline, chlortetracycline and oxytetracycline, respectively)). DKA stock solutions were composed of six DKA species with equal weight concentrations and equal volumes of each species. Zebrafish were continuously exposed to DKAs from embryos (2 hpf) to larvae (5 dpf) stage. The DKA stock solution was prepared by dissolving the six DKA species in ultra-pure water using 0.1 M NaOH as cosolvent; stock solutions were replaced daily to maintain a constant concentration.

### 2.3. Total RNA extraction

The 72- or 120-hpf zebrafish embryos in each treatment group were collected and rinsed using phosphorous buffer solution (PBS, Solarbio, Beijing, China) in an RNase-free 1.5 mL EP tube. Total RNA was isolated according to the protocol reported by Wu and coworkers (Wu et al., 2015).