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Short communication

Amikacin-induced Fin Reduction is Mediated by Autophagy

I-Ting Tsai[1#], Ying-Hsin Chen[2#], Yau-Hung Chen[*1], and Yun-Hsin Wang[*3]

[1] Department of Chemistry, Tamkang University, 151, Yingzhuan Road, Danshui Dist., New Taipei City 25137, Taiwan, ROC
[2] Department of Emergency Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
[3] Division of Basic Research, Koo Foundation Sun Yat-Sen Cancer Center, Taipei, Taiwan

Abstract: Despite its medical use, little is known about the mechanisms underlying amikacin-induced embryotoxicity, including fin reduction, in zebrafish. In this study, we examined the expression of well-known autophagy markers mTOR (target of rapamycin), *atg10* (autophagy-related gene), *atg12* and LC3 (mammalian homolog of Atg8) in amikacin-treated zebrafish embryos. Our results indicated that the mRNA expression level of *atg12* in the amikacin-treated group was significantly increased by 1.5-fold ($p<0.05$) compared with the corresponding mock control group, while the expression levels of *atg10* and mTOR were significantly decreased by 0.74-fold ($p<0.05$) and 0.58-fold ($p<0.05$), respectively. Western blot analysis revealed that LC3 protein expression was induced by amikacin. Taken together, these data suggest that amikacin-induced fin reduction is mediated by fin cell autophagy. (DOI: 10.1293/tox.26.79; J Toxicol Pathol 2013; 26: 79–82)

Key words: amikacin, autophagy, embryotoxicity, fin reduction, zebrafish

Amikacin is an aminoglycoside antibiotic that is commonly used to treat bacterial infections[1–3]. Although amikacin has been Food and Drug Administration (FDA)-approved for human use, its use has been associated with potential risks, including hearing loss and liver and renal damage[4–6]. In addition, amikacin treatment has been shown to lead to ototoxicity, hepatotoxicity, and nephrotoxicity in rodents and to result in fin reduction and neuromast loss in zebrafish[7–9]. These observations indicate that amikacin-induced toxicity is conserved among many species. In light of this fact, it is important to study the molecular mechanisms underlying amikacin-induced toxicological processes.

In rats, aminoglycoside-induced nephrotoxicity has been shown to be due to apoptosis in the renal proximal tubules, and aminoglycoside-induced ototoxicity has been linked to hair cell apoptosis[10]. In addition to apoptosis, autophagy has been shown to play an important role in aminoglycoside-induced ototoxicity in mouse models[6]. Autophagy is an evolutionarily conserved, lysosome-mediated degradation and is involved in the regulation of cell survival, differentiation, and death[11,12]. More recently, it was discovered that autophagy can be induced by starvation or drug treatment[13–16]. Furthermore, we have previously shown that zebrafish embryos displayed fin reductions following treatment with 10 ppm amikacin, including dorsal, ventral and pelvic fin shrinkage or absence[9]. Here, we followed the same exposure protocol (Fig. 1) and used live video to dynamically observe the teratogenic process of fin reduction following amikacin treatment. As shown in Fig. 2, vacuoles appear on the fins of amikacin-treated embryos [10 ppm, from 48 to 74 hours post fertilization (hpf)]. Pictures were taken sequentially from a 12-min live video (Suppl Movie: on-line only), and these pictures showed that the vacuoles appeared randomly in the fin and that they then gathered together to consequently cause fin reduction (Figs. 2A–2F).

What causes fin reduction? The appearance of vacuoles on fin cells seems to be the first sign of amikacin-induced fin reduction. However, vacuolization has been reported to be induced by many factors, including oxidative stress,

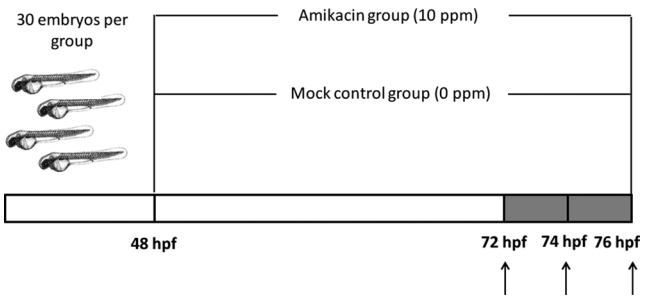

Fig. 1. Schematic representation of experimental protocols performed in this study. Live video analysis of zebrafish fins was performed after 72–76 hpf, whereas immunostaining, real-time PCR and Western blotting were conducted at 74 hpf.

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[#] I-T Tsai and Y-H Chen contributed equally to this work.
*Corresponding authors: Y-H Chen (e-mail: yauhung@mail.tku.edu.tw), Y-H Wang (e-mail: 129180@mail.tku.edu.tw)

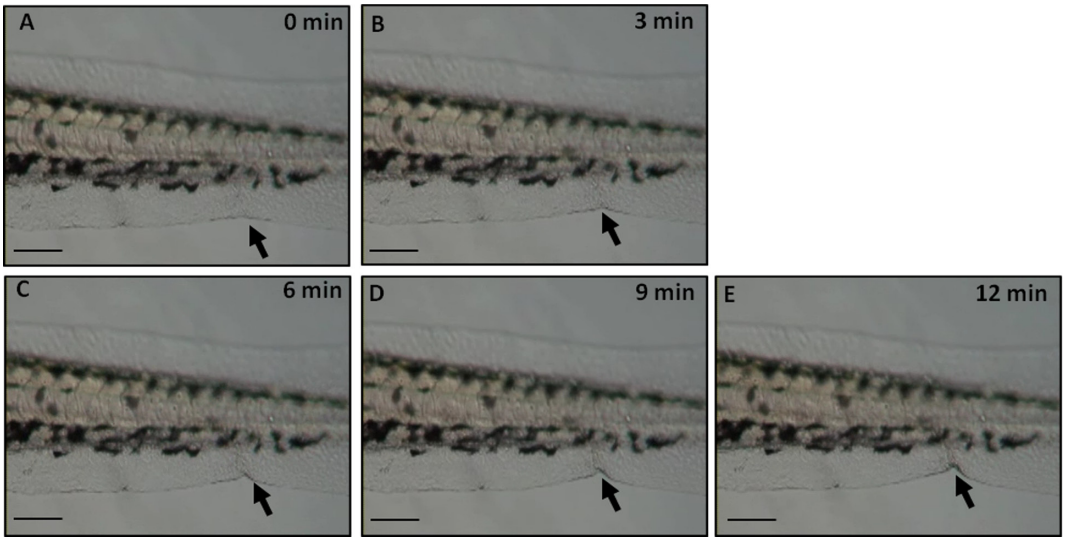

Fig. 2. Fin reductions in zebrafish embryos after amikacin treatment. Images were captured from live videos of zebrafish embryos after treatment with 10 ppm amikacin. (A) Initial position of the vacuole in the fin cells. (B–E) Position of the vacuole 3–12 min later. Arrow indicates the position of the vacuole. Scale bar: 0.2 mm

Table 1. Primers Used in This Study

Primer	Sequence (5' → 3')
β-actin F	CAGCAAGCAGGAGTACGATGAGT
β-actin R	TTGAATCTCATTGCTAGGCCATT
ATG10 F	ATGACCGGTGAGAGAAAGCCT
ATG10 R	AGCCTTCATCAGAGCCCTTGA
ATG12 F	TATGTTAATCAGTCGTTTGCGCC
ATG12 R	CTAGTTTGCCGTCACTTCCGA
mTOR F	TTATCGTGCTGGTCCGAGCT
mTOR R	AAGTGGGCCCTTATCGCTGT

autophagy, and drug treatment[17–19]. Our previous study showed that TUNEL-positive staining did not appear on fin mesenchymal cells. We therefore concluded that apoptosis is not the main cause of fin reduction[9]. Taken together with the observations from the live video analysis (Fig. 2), we assumed that amikacin treatment may activate other cell death pathways and consequently cause fin reduction.

Autophagy consists of several sequential steps, including initiation, autophagosome formation, autophagosome-lysosome fusion and degradation. Experimental evidence accumulated from previous studies has shown that many genes and proteins contribute to each specific step of autophagy. For example, the autophagy-related gene (*atg*) Atg10 (protein) is known to be essential for the formation of the Atg12-Atg5 conjugate, the formation of which is essential to autophagosome formation. Inhibition of mTOR (mammalian target of rapamycin) is known to be important during the initiation step of autophagy[20–23]. To test whether amikacin-induced fin reduction is due to autophagy, we first compared the expression levels of *atg10*, *atg12* and mTOR between mock control and amikacin-treated groups (100 embryos each group) using real-time PCR (all primer sequences are listed in Table 1). As shown in Fig. 3, the mRNA

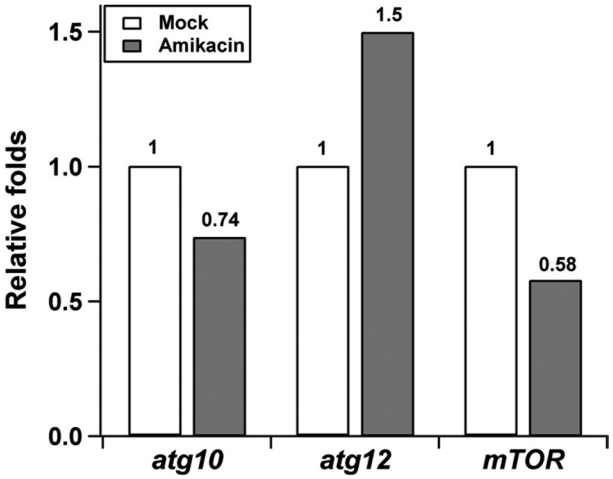

Fig. 3. Relative quantification of mRNA expression using the comparative C_T method. One hundred embryos derived from the mock control (0 ppm) and amikacin-treated (10 ppm) groups were collected, and their total RNA were isolated using a standard procedure. Approximately 25 μg of total RNA from each group was used for cDNA synthesis; 1% of cDNA was used for each quantitative PCR reaction. Quantitative PCR was performed under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C using 2X Power SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of forward and reverse primers (Table 1). Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicate, and the fold-changes in expression were derived using the comparative CT method. β-actin was used as the endogenous control for relative quantification. The amikacin-treated group was significantly different from the corresponding mock group (C_T: cycles of qPCR; relative folds to control group = $2^{-\Delta\Delta CT}$).

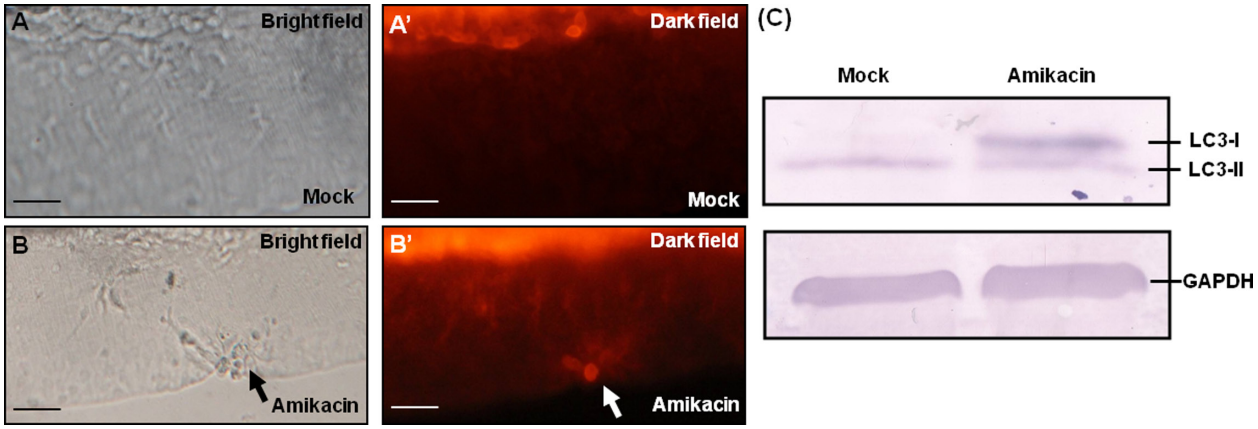

Fig. 4. Effects of amikacin on fin cells. Embryos derived from the mock control (A, A') or amikacin-treated groups (B, B') were stained with antibodies against LC3. (C) Results of Western blot analysis of the mock control and amikacin-treated embryonic lysates using antibodies against LC3 and GAPDH. Scale bar: 0.1 mm

levels of *atg12* in the amikacin-treated group were significantly increased by 1.5-fold ($p<0.05$) compared with the corresponding mock control group. In contrast, the mRNA levels of *atg10* and mTOR in the amikacin-treated group were significantly decreased by 0.74-fold ($p<0.05$) and 0.58-fold ($p<0.05$), respectively, compared with the corresponding mock control group. These observations revealed that amikacin affected fin morphology by mediating the gene expression of mTOR, *atg10* and *atg12*.

The LC3 protein is the mammalian homolog of Atg8. The cytosolic form of LC3-I has been shown to be cleaved to form the membrane form of LC3-II during the induction of autophagy. These two proteins have been used as markers to study autophagy[23]. Thus, we monitored the expression of LC3-I and LC3-II in the fin reduction zebrafish embryos by performing Western blotting and immunofluorescence analyses. Western blotting and antibody labeling were performed as previously described[24–30] using the following antibodies: LC3 (autophagosomal marker; 1:200, Novus, Littleton, CO, USA) and GAPDH (loading control; 1: 1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The results indicated that the LC3-positive cells co-localized with the vacuoles in the amikacin-treated embryos (Figs. 4A and 4A' vs 4B and 4B'). Western blot analysis revealed that the expression levels of LC3-II remained unchanged, but the expression levels of LC3-I were upregulated (Fig. 4C).

In this study, we showed that decreases in the expression levels of mTOR and the accumulation of LC3-I correspond with the reported gene expression profile of the initiation step of autophagy[20–23]. These observations revealed that amikacin affected fin morphology by mediating the expression of mTOR and LC3-I, and consequently lead to the initiation of autophagy. In addition, our data showed that the expression of an autophagosome marker, *atg10*, is decreased following amikacin treatment, which is inconsistent with well-known gene expression profiles during autophagosome formation. This inconsistency might be due to (1) amikacin being mainly involved in the initiation of autophagy, but not autophagosome formation, or (2) amikacin possibly regulating *atg10* expression at the protein level. Moreover, a transgenic zebrafish line (LC3:GFP) has been created that should provide a convenient tool for assaying amikacin-induced autophagic activity during embryogenesis *in vivo*[27]. In conclusion, amikacin-induced fin malformations can easily be dynamically observed *in vivo* using the present model, which may provide novel insights into the adverse effects of amikacin.

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