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Stereoselective induction of developmental toxicity and immunotoxicity by acetochlor in the early life stage of zebrafish

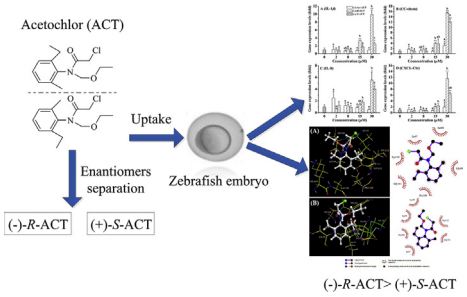
Chao Xu [a], Wenqing Tu [a, b, *], Mi Deng [b, c], Yuanxiang Jin [a], Bin Lu [a], Chaonan Zhang [a], Chunmian Lin [a], Yongming Wu [b], Weiping Liu [d]

[a] College of Environment, Zhejiang University of Technology, Hangzhou 310032, China
[b] Research Institute of Poyang Lake, Jiangxi Academy of Sciences, Nanchang 330029, China
[c] Key Laboratory of Poyang Lake of Ministry of Education, Nanchang University, Nanchang 330031, China
[d] College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, China

HIGHLIGHTS

- Stereoselective induction of developmental toxicity and immunotoxicity by ACT in zebrafish embryos was investigated.
- (-)-*R*-ACT showed stronger effects than (+)-*S*-ACT in zebrafish developmental toxicity endpoints.
- ACT exposure significantly increased the levels of Il-1b protein expression in all (-)-*R*-ACT treatment groups.
- Both *in vivo* and *in silico* studies uniquely disclosed the enantioselective immunotoxicity of ACT in fish.

GRAPHICAL ABSTRACT



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ABSTRACT

Acetochlor (ACT) has been frequently detected in the aquatic environment and implicated in disruption of the immune system in fish, the mechanisms of which, especially at enantiomeric levels, remains unclear. In the present study, embryonic zebrafish were exposed to ACT and its enantiomers at concentrations of 0, 2, 8, 15, 30 and 60 μM from 2 h post-fertilization (hpf) to 72 hpf. We demonstrated that ACT and its enantiomers could cause time- and concentration-dependent mortality (72 h LC_{50} ranged from 48.4 to 53.1 μM) and developmental malformations (*e.g.*, 48 h EC_{50} for yolk sac edema ranged from 36.7 to 54.1 μM), as well as increase transcription of the key genes involved in the innate immune system. A consistent enantioselectivity in these endpoints was observed with (−)-*R*-ACT showed stronger effects than (+)-*S*-ACT, and the transcription levels of *il-1β* exhibited significant enantioselectivity at concentrations as low as 8 μM. Further Western blot analysis revealed that significant elevations of Il-1β protein expression in all (−)-*R*-ACT treatment groups. According to the molecular docking and molecular dynamics simulations, the enantioselectivity between ACT enantiomers was attributed to the distinct binding affinity to Il-1β. Overall, our *in vivo* and *in silico* studies uniquely disclosed the enantioselective immunotoxicity of ACT and its underlying mechanisms and highlighted the need to evaluate the environmental risk of chiral chloroacetamide herbicide in aquatic organisms at enantiomeric levels.

* Corresponding author. Research Institute of Poyang Lake, Jiangxi Academy of Sciences, Nanchang 330029, China.
E-mail address: twq0228@163.com (W. Tu).

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1. Introduction

Chloroacetamide herbicides have been widely used to control annual grasses and broad leaf weeds. However, the widespread occurrence of these compounds in different environmental compartments has drawn considerable attention to their detrimental effects on wildlife and humans in recent years (Coquillé et al., 2015; Saha et al., 2012; Tu et al., 2013). Acetochlor (ACT) is one of the most frequently detected chloroacetamide herbicide in the aquatic environment. It has been reported that more than 10 million kg of ACT have been used annually in China (Li et al., 2015).

The widespread use of ACT in agricultural fields resulted in pollution in adjacent surface water. Studies have shown that ACT residue concentrations ranged from 0.05 to 2.5 μg/L in surface water (Boyd, 2000; Kolpin et al., 1996), potentially causing adverse effects toward different aquatic organisms. ACT has been classified as a B-2 carcinogen by the U.S. Environmental Protection Agency (USEPA, 2000, 2006, 2007). Previous studies have shown that ACT may have genetic toxicity (Coleman et al., 2000), induce oxidative stress and apoptosis (Jiang et al., 2015) and alter thyroid hormone levels (Li et al., 2009; Yang et al., 2016).

Previous studies have revealed that the immune system is one of the most sensitive targets for many environmental contaminants (Jiang et al., 2016; Jin et al., 2011, 2015). The adverse effects of environmental chemicals on the immune system in aquatic organisms, such as fish may affect their survival, development and growth (Xu et al., 2013). In fish, the immune system consists of complex and highly specialized cells, tissues, and organs, and is classically divided into innate and adaptive immunity. Cytokines are potent, multifunctional, pleiotropic proteins and play a major role in the innate and adaptive immune system against viral infection.

Our previous study showed that butachlor, another chloroacetamide herbicide, could upregulate the expression of cytokines such as *il-1β*, *cc-chemokine* and *il-8* in zebrafish embryos (Tu et al., 2013). Since ACT possesses the similar chemical structure to butachlor, it might be hypothesized that ACT may share the same mechanism of toxicity with butachlor and exhibit adverse effects on the immune system. Moreover, a recent study has indicated that ACT significantly regulated the transcriptional levels of the genes involved in the immune system of zebrafish (Jiang et al., 2015). However, to date, the molecular mechanism by which ACT generates immunotoxicity has not been fully elucidated and further studies are required to understand the details of the interaction between ACT and immune-related proteins.

Like many chiral pesticides, ACT is an axially chiral compound and has a pair of enantiomers, (−)-*R*-ACT and (+)-*S*-ACT [Supporting Information (SI) Fig. S1]. Enantiomers of chiral pesticides are known to interact selectively with biological systems and lead to distinct toxicological effects, highlighting the importance of considering enantioselectivity in the ecotoxicological assessment for chiral pesticides. To date, the chiral separation and enantioselective environmental behavior of chloroacetamide herbicides have been studied (Garrison, 2006; Liu et al., 2015). However, studies on the enantioselectivity of ACT in developmental toxicity and immunotoxicity are still limited. Due to the frequent occurrence and persistence of ACT in water, it is essential to evaluate the enantioselectivity in aquatic toxicity.

The embryonic life stages of vertebrates such as fish are uniquely susceptible to environmental contaminants (Deng et al., 2009). Zebrafish with its small, well established genetic and genomic information and transparent embryo has emerged as a predominant model for evaluating developmental toxicity and immunotoxicity (Jiang et al., 2016; Jin et al., 2010). In this study, zebrafish embryo was used as an *in vivo* model for the evaluation of the aquatic toxicity of ACT. The enantioselectivity of ACT in developmental toxicity, the protein and mRNA expression levels of innate immune system-related genes were explored. Meanwhile, molecular docking analysis and molecular dynamics simulations were used for further investigation of enantioselective mechanism. Our *in vivo* and *in silico* studies will be useful for improving the understanding of the enantioselectivity of ACT on the fish immune system.

2. Materials and methods

2.1. Chemicals

Acetochlor (>95% purity, (−)-*R*-ACT/(+)-*S*-ACT = 1:1) was purchased from Sigma-Aldrich (St. Louis, MO, USA). ACT enantiomers were baseline separated on a high-performance liquid chromatography (HPLC) system (Jasco, Tokyo, Japan) (Fig. S2) with the enantiomeric purities >99% and their absolute configurations have been successfully assigned (Gu et al., 2011). A detailed procedure for enantiomer separation is provided in the SI (Text S1). The stock solutions of ACT and its enantiomers were prepared in *n*-hexane at 1000 mg/L and diluted with embryos medium (EM) (Westerfield, 1995) just before use. The final concentration of *n*-hexane in working solutions did not exceed 0.1% (*v*/*v*). All other chemicals/solvents used in this study were of analytical grade.

2.2. Zebrafish maintenance and experimental design

Adult zebrafish (AB strain) maintenance and embryo production were carried out as described previously (Tu et al., 2016a). All procedures involving animals were approved by the Independent Animal Care Committee of Zhejiang University of Technology.

2.2.1. Exposure for survival and malformation analysis

Following the OECD guidance for the Fish Embryo Toxicity (FET) Test (OECD, 1998), the concentrations of ACT and its enantiomers at concentrations of 0, 2, 8, 15, 30, 60 μM were chosen. Normally fertilized embryos (2 h post-fertilization, hpf) were randomly transferred to 24-well plates (three embryos per well) containing 2 mL of exposure solution. ACT-free EM was used as blank control and the solvent blank contained 0.1% *n*-hexane. All treatments were replicated three times. The plates were covered and then incubated in a light incubator at 28 ± 1 °C with a 14 h:10 h light:dark cycle. The exposure experiments lasted for 72 hpf and the exposure solutions were renewed daily with freshly prepared solutions. The embryonic development was monitored daily under an inverted dissecting microscope (Leica Microsystems, Wetzlar, Germany). Dead embryos were identified by failure of the heartbeat or by somite formation with a non-detached tail and removed in a timely manner. The malformations, including yolk sac edema (YSE), pericardial edema (PE) and crooked body (CB) were checked daily and the percentage of malformations was calculated as the ratio of the number of malformed individuals to the total number of embryos.

2.2.2. Exposure for biochemical analysis

Based on the recommended application dose of ACT used in paddy fields and the results from the acute zebrafish embryo test, the concentrations of ACT and its enantiomers were 0, 2, 8, 15 and 30 μM. The exposure protocol was conducted according to our previously described (Tu et al., 2016a). Briefly, normally fertilized embryos (2 hpf) were distributed randomly in 1 L glass beakers containing 500 mL of exposure solution. Each treatment group contained three beakers with 150 embryos per beaker. Since the innate immune system is active from spawning period and acts as the only tool in defense against infection during the first three days