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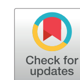
Fluazinam impairs oxidative phosphorylation and induces hyper/hypo-activity in a dose specific manner in zebrafish larvae



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HIGHLIGHTS

- Fluazinam is a pyridinamine fungicide that affects mitochondria and is neurotoxic.
- Fluazinam decreased basal and ATP-linked respiration in zebrafish embryos.
- *Sod2* and *hsp70* mRNA levels were increased by 0.5 μM fluazinam.
- *Tyrosine hydroxylase 1* and *dopamine receptor 2a* mRNA were decreased by fluazinam.
- Fluazinam induced hyper- and hypo-activity in larval fish, a response that was dose-specific.

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ABSTRACT

Fluazinam is a pyridinamine fungicide that induces oxidative stress and mitochondrial damage in cells, and it has been reported to be neurotoxic. To characterize the biological effects of fluazinam, we assessed mitochondrial bioenergetics, dopamine system expression, and behavior of early life staged zebrafish (0.01 μM–0.5 μM). Fluazinam at environmentally-relevant levels did not induce sub-lethal effects in larvae, but at the LC_{50} (0.5 μM), fluazinam decreased basal and ATP-linked respiration significantly in embryos. As mitochondria are directly related to redox homeostasis and apoptosis, the expression of genes related to oxidative stress and apoptosis were measured. *Superoxide dismutase 2* (*sod2*), *heat stock protein 70* (*hsp70*), *bcl2-associated X protein* (*bax*), and *caspase 9* (*casp9*) mRNA levels were up-regulated by 0.5 μM fluazinam. Taken together, there was evidence for mitochondrial dysfunction and oxidative damage at the highest concentration of fluazinam (0.5 μM) tested. As there are reports for fluazinam-induced neurotoxicity in dopamine synthesizing cells, transcriptional targets in the dopamine system were assessed in the zebrafish. *Tyrosine hydroxylase 1* (*th1*) and *dopamine receptor 2a* (*drd2a*) mRNA levels were decreased by 0.5 μM fluazinam, suggesting that this fungicide may affect the dopaminergic system. To further assess the potential for fluazinam-mediated neuromodulation, the dark photokinesis response was assessed in larvae following exposure. Larvae exposed to 0.1 μM fluazinam showed hyperactivity, while larvae exposed to 0.2 and 0.3 μM showed hypo-activity. This study demonstrates that fluazinam disrupts mitochondrial bioenergetics in zebrafish, inducing an oxidative stress response, and aberrant behaviors in larvae that are dose dependent.

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1. Introduction

Pesticides used to protect crops from pests and fungi can have detrimental effects on non-target organisms. Fluazinam is a pyridinamine fungicide used for the control of mold on crops. This fungicide works as an uncoupler of mitochondrial oxidative

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phosphorylation in cells through direct inhibition of ATP synthase (Guo et al., 1991; Vitoratos, 2014). Fluazinam has been listed in the ToxCast™ Phase I chemicals (http://epa.gov/ncct/toxcast/) as one with potential environmental concerns as it is considered to be very highly toxic to aquatic organisms (US EPA. 2001; EFSA, 2008). Products for example containing fluazinam are labelled to disallow aerial or irrigation system application, in case of drift, contamination into water sources, and exposure to non-target organisms. Despite restrictions on application, fluazinam can still be released into the aquatic environment because of its use in agricultural areas (Rijtema et al., 1999; van Wijngaarden et al., 2004; Wang et al., 2016). A study by Räsänen et al. (2015)investigated the relationship between freshwater ecotoxicology impact and different pesticides and determined that fluazinam was the most impactful and hazardous of all pesticides analyzed due to its high toxicity to animals. Despite this however, there are few data reporting measured concentrations of this fungicide in aquatic environments. Based on maximum application rates, this fungicide was estimated to reach a peak concentration value of 18 μg/L in surface water, and has a 56-day chronic averaged concentration value of 3.15 μg/L (US EPA. 2001). Additionally, van Wijngaarden et al. (2004) performed an indoor freshwater microcosm study based on a realistic application scenario in tulip cultivation, and calculated fluazinam residue in aquatic microcosm to be 0.27–6.7 μg/L. There are few toxicological investigations into the mechanisms of this compound, and continued diligence is needed to ascertain whether this chemical poses a risk to organisms in aquatic environments.

Fluazinam is reported to be very highly toxic to fish. These data come from 96-h acute toxicity tests using adult fish in a variety of fish species (*Onchorhynchus mykiss*, *Cyprinodon variegatus*, *Brachydanio rerio*, *Lepomis macrochirus*, *Poecilia reticulata*) and LC_{50} values range from 36 μg/L to 120 μg/L, while no observed effect concentration (NOEC) values range from 15 μg/L to 80 μg/L (Hill, 1985; Gelin and Laveglia, 1992, 1993; Shults et al., 1993; Peither, 2001a, 2001b). It was also reported that rainbow trout (*Onchorhynchus mykiss*) were observed to exhibit some behavioral and sub-lethal effects such as pigmentation, lethargy or loss of equilibrium with an acute exposure to 28 μg/L and 33 μg/L fluazinam for 96 h (Gelin and Laveglia, 1992). In the same species, fluazinam was also proposed to result in surfacing and rapid respiration rates in the fish at the level of 57 μg/L (Hill, 1985). Similar effects were observed as well in bluegill sunfish (*Lepomis macrochirus*) at 34 μg/L fluazinam (Gelin and Laveglia, 1993), and in zebrafish (*Brachydanio rerio*) at 49 μg/L fluazinam (Peither, 2001a). Additionally, this fungicide is reported to be disruptive to fathead minnow (*Pimephales promelas*) during early life stage exposure, and NOEC value for fish survival at hatching was as low as 10 μg/L (Fillmore and Laveglia, 1993). Given these data, and the report that fluazinam can accumulative in fish tissues (US EPA. 2001), the potential toxicity of fluazinam at sub-lethal concentrations remains an important question.

Fluazinam is a potent uncoupler in mammalian mitochondria and acts through protonation/deprotonation of its amino group (Guo et al., 1991). This fungicide can exhibit specific effects on energy production directly and can inhibit oxidative phosphorylation in cells (Leroux, 1996). The fungicide is proposed to target mitochondrial complex I to induce reactive oxygen species (ROS) in human neuroblastoma SH-SY5Y cells (Lee et al., 2011; Lee et al., 2012). These cells are used as a neurotoxicity model to determine the effects of chemicals on dopamine production. Additionally, Lee et al. (2012) also reported cytochrome *c* release and caspase 3 activation through p38 and JNK signaling following fluazinam exposure, suggesting that the mitochondrial apoptotic pathway is involved in fluazinam-induced neurotoxicity. Other agricultural fungicides such as maneb, benomyl, carboxin and flutolanil are also suspected to cause dopaminergic neurodegeneration through disruption of mitochondrial function (Leroux, 1996; Zhang et al., 2003; Fitzmaurice et al., 2013); thus there is a link between pesticide exposure, mitochondrial dysfunction, and dopaminergic cell death. Moreover, mitochondria are major sites of energy production in cells and the deficits in energy production can result in neurodegenerative disorders and locomotor deficits (DiMauro and Schon, 2008; Coskun et al., 2012; Steele et al., 2014). Therefore, studies into the toxic effects of fluazinam on dopaminergic signaling and locomotor activity are also relevant.

The objectives of this study were to determine mechanisms of developmental toxicity of fluazinam to zebrafish embryos and larvae. Developmental toxicity has previously been observed in larval zebrafish following fluazinam exposure. Padilla et al. (2012) measured chemical potencies for a number of compounds in a Concentration-Response study, determining half-maximal activity concentrations (AC_{50}) for many chemicals. The AC_{50} for fluazinam in the zebrafish assay was determined to be 0.85 μM. Here, zebrafish embryos were exposed to environmentally relevant concentrations of fluazinam (0.001, 0.01, 0.1 μM or 0.465, 4.65, 46.51 μg/L), up to the medium lethal concentration (LC_{50}) of fluazinam (0.5 μM or 232.54 μg/L) as determined by us in pilot studies. We observed developmental deficits in zebrafish embryos, and measured mitochondrial oxygen consumption rates of embryos. We also measured transcript levels of genes related to the oxidative stress response and apoptosis, as the ROS and apoptotic pathway are involved in mitochondrial dysfunction. Additionally, we measured transcripts associated with dopaminergic signaling, as the responses of dopaminergic system are putative mechanisms that could in part underlie any behavioral deficits observed in larvae. Data are not available for fluazinam in terms of mechanisms of action for developing aquatic organisms, and this study addresses this current knowledge gap.

2. Materials and methods

2.1. Experimental design

Fluazinam (CAS: 79622-59-6, purity > 98.5%, Sigma, USA) stock solutions were prepared in dimethyl sulfoxide (DMSO), and diluted into embryo rearing medium (ERM), to achieve the final nominal concentrations of the chemical (0.01% DMSO). Pharmaceutical agents used for oxygen consumption rate measurements (i.e. oligomycin, FCCP and sodium azide) were purchased from Sigma-Aldrich Co. LLC (USA). Oligomycin and FCCP were prepared in DMSO, and sodium azide was prepared in ERM.

Adult wildtype zebrafish (*Danio rerio*) were housed in the Cancer Genetics Research Center of University of Florida. The University of Florida Institutional Animal Care and Use Committee approved the experimental protocols, and experiments adhere to all relevant National Institutes of Health guidelines on the use of animals. Embryos in the control group were incubated in 0.01% DMSO only, and the treatment groups were exposed to one dose of either 0.001, 0.01, 0.1 μM fluazinam and the 96-h LC_{50} of 0.5 μM of fluazinam. To determine the LC_{50} of fluazinam, an initial group of embryos were exposed from 6 h post fertilized (hpf) for 96 h and mortality was recorded every 24 h (N = 24). The dose-response relationship curve was generated using Graph Pad Prism 6.0 (Graph Pad Software, San Diego, CA, USA).

In the following experiments, embryos were treated with one dose of either 0.001, 0.01, 0.1, 0.2, 0.3 or 0.5 μM fluazinam at 28 ± 1 °C. The chemical was prepared as per above and renewed daily at the same time of day. Responses in one assay guided us to focus on specific concentrations in other assays, thus not every dose was tested in every assay. For developmental profiling, 96 embryos at ~6 hpf were transferred into a 96-well plate for 96h control and

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