SDHI Fungicide Toxicity and Associated Adverse Outcome Pathways

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Succinate dehydrogenase inhibitor (SDHI) fungicides are increasingly used in agriculture to combat molds and fungi, two major threats to both food supply and public health. However, the essential requirement for the succinate dehydrogenase (SDH) complex—the molecular target of SDHIs—in energy metabolism for almost all extant eukaryotes and the lack of species specificity of these fungicides raise concerns about their toxicity toward off-target organisms and, more generally, toward the environment.

succinate dehydrogenase inhibitors | SDHIs | fungicide | zebrafish | bixafen | boscalid | fluxapyroxad | flutolanil | isoflucypram | isopyrazam | penthiopyrad | sedaxane | thifluzamide | neurodevelopment

1. Introduction

Molds and fungi have been a major threat to crops throughout human history. A potato crop fungus caused the Irish potato famine of the 1850s, and mildew mold wrought the complete destruction of French vineyards at the end of the 19th century. Besides crop destruction, fungi also produce mycotoxins, which can contaminate agricultural products and make them unfit for consumption or even toxic, as exemplified by the mass food poisoning in Kenya in 2004 due to the consumption of moldy maize contaminated with aflatoxin, which caused 125 deaths [1]. From the 1950s onward, the chemical industry has developed fungicides to respond to the threats posed by these pests. These agents have since become increasingly widely used in modern agricultural practices worldwide. Today, among the different classes of fungicides, SDHIs are the fastest growing family as seen by the number of new products arriving on the market over the past 10 years [2]. Their fungicidal properties rely on their ability to inhibit the SDH/electron transport chain (ETC) complex II (CII), an essential and evolutionarily conserved mitochondrial enzymatic complex critically required for proper functioning of both the ETC and tricarboxylic acid (TCA)/Krebs cycle, both essential for cellular energy production and ATP synthesis [3].

Importantly, the SDH/CII complex is crucial for energy production not only in fungi, but also in all organisms that have mitochondria (i.e., almost all extant eukaryotes). It was recently shown that eight SDHI fungicides currently used for agricultural purposes are highly efficient inhibitors of SDH activity in off-target species, such as bees, earthworms, and humans [4]. These findings raise concerns about the possible toxicity of these compounds toward non-target organisms and, more generally, toward the environment. According to the Pesticide Properties

DataBase of the International Union of Pure and Applied Chemistry (IUPAC), SDHI fungicides display low acute toxicity toward mammals and birds, with acute oral LD_{50} values ranging from 2000 mg/kg to over 6500 mg/kg in rodents and about 2500 mg/kg in birds. SDHIs can be highly toxic to fish, with 96 h LC_{50} values for the adult common carp (*Cyprinus carpio*) as low as 8.7 nM, 70 nM, and 170 nM for benzovindiflupyr, isopyrazam, and isoflucypram, respectively. According to manufacturers' recommendations, the concentrations of SDHI solutions spread on fields should range from 0.5 to 2 mM, and the initial concentration of thifluzamide in paddy water after spraying is 7.4 µM [4]. In addition, the increasing use of SDHI fungicides worldwide results in these substances being frequently detected in aquatic environments [5], sometimes at concentrations exceeding reported toxic levels (e.g., 0.72 µM flutolanil in effluent water in Japan [6] and 0.1 µM boscalid in coastal estuary water in California [7]).

Over the last two decades, the zebrafish, a small, easily bred freshwater fish, has become increasingly used as a model in many fields of biology, including toxicology. The reasons for the popularity of this vertebrate model are numerous and well-known. They have already been described in many reviews [8] and so will not be discussed here. Suffice to say that the zebrafish has been recommended by the OECD as a model organism to study the toxicity of environment-contaminating chemicals and pesticides [9].

2. The SDHI Fungicides—A Fast-Growing Pesticide Family Who's Toxicity Has Been Scantly Studied

The fungicidal property of SDHIs relies on their ability to inhibit the SDH/CII complex in molds and fungi. This complex is a universal key component of the mitochondrial respiratory chain, which transfers electrons generated during the oxidation of succinate to fumarate to a pool of ubiquinone, which is then reduced to ubiquinol [10]. The SDH/CII complex is therefore crucial for the proper functioning of both the mitochondrion ETC and TCA cycles—two metabolic pathways that are essential for energy supply, cell metabolism, and many other vital processes. Hence, even partial inhibition of SDH activity is expected to cause marked changes in metabolism and have severe adverse consequences for the cells [11]. Importantly, besides the essential and evolutionarily conserved requirement for the SDH/CII complex, the four proteins constituting the enzymatic complex (SDHA to D) and, especially, the quinone binding pocket of the tetrameric complex, which is the molecular target of all carboxin-derived SDHIs, display a high level of evolutionary conservation at both the structural and amino acid sequence levels [4]. As a likely consequence of this conservation of SDH proteins throughout evolution, it has also been shown [3] that eight SDHI fungicides currently used in agriculture are efficient inhibitors of the SDH activity in several off-target species, including bees, earthworms, and humans, with IC_{50} values toward human SDH as low as 0.34 and 0.63 µM in the case of SDHIs bixafen and isopyrazam, respectively.

The first SDHI fungicide, carboxin, was introduced in 1969 to combat basidiomycete fungi, such as rusts and smuts (IUPAC, Pesticide Properties DataBase). However, Mowery et al. [12] investigated the effect of this substance on the activity of SDH extracted from beef heart and demonstrated that carboxin efficiently inhibited this SDH enzymatic activity, with IC_{50} values in the micromolar range. Alongside carboxin and flutolanil, which were first marketed over 30 years ago, a new generation of SDHI fungicides has appeared in the last ten years comprising boscalid, benzovindiflupyr, isopyrazam, penthiopyrad, sedaxane, fluopyram, and others. In 2021, national and

international regulatory authorities approved 22 SDHI fungicides worldwide, and 2 are pending authorization (FRAC, 2021, classification of fungicides). In addition, the emergence of resistance to existing SDHIs makes the discovery of fungicides with novel modes of action an urgent need, which should also lead to an increase in the number of new SDHIs in the near future [12]. Of particular importance here, some new-generation SDHI fungicides display biocidal activities that go beyond mold destruction, as illustrated by fluopyram, which is also used as a highly effective nematicide to combat parasitic nematodes in soils and lawns [13]. It is of note that fluopyram caused an increased incidence of thyroid follicular cell adenomas in male mice at 105 mg/kg/day in a mouse oncogenicity study [14]. However, the toxicity of fluopyram toward zebrafish embryos, larvae, or adults has not yet been evaluated.

3. Acute Toxicity of SDHI Fungicides

According to the IUPAC Pesticide Properties DataBase, most SDHI fungicides are considered as moderately toxic to fish species; the rainbow trout (*Oncorhynchus mykiss*), the fathead minnow (*Pimephales promelas*), and the common carp have 96 h LC_{50} values of >1 µM. However, four SDHIs display high toxicity to adult fish, namely, benzovindiflupyr, isopyrazam, isoflucypram, and bixafen (96 h LC_{50} 8.7 nM, 70 nM, 170 nM, and 230 nM, respectively) (**Table 1** and **Table 2**).

Table 1. LC_{50} values of SDHIs determined in zebrafish.

SDHIs	96 h LC_{50}	Stages
Bixafen	2.12 µM	embryo
	2.7 µM	embryo
Boscalid	7.72 µM	embryo
	4.85 µM	adult
Flutolanil	16.91 µM	embryo
	12.65 µM	larvae (144 hpf)
	12.09 µM	larvae (84 hpf)
	8.35 µM	adult

SDHIs	96 h LC_{50}	Stages
Fluxapyroxad	1.83 µM	larvae
	2.4 µM	adult
	3.64 µM	embryo
Isopyrazam	0.14 µM	embryo
Penthiopyrad	7.70 µM	embryo
	6.62 µM	larvae
Sedaxane	11.7 µM	embryo
Thifluzamide	7.93 µM	adult
	6.66 µM	larvae
	5.83 µM	embryo

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SDHIs	96 h LC_{50}	Species
Benodanil	19.8 µM	*Oncorhynchus mykiss*
Benzovindiflupyr	8.7 nM	*Cyprinus carpio*
Bixafen	0.23 µM	*Oncorhynchus mykiss*
Boscalid	7.86 µM	*Oncorhynchus mykiss*
Fenfuram	54.66 µM	*Poecilia reticulata*
Fluindapyr	0.34 µM	unknown species

SDHIs	96 h LC_{50}	Species
Fluopyram	2.47 µM	*Coleonyx variegatus*
Flutolanil	16.7 µM	*Lepomis macrochirus*
Fluxapyroxad	0.76 µM	*Cyprinus carpio*
	1.22 µM	*Pimephales promelas*
	3.02 µM	*Lepomis macrochiris*
	1.43 µM	*Oncorhynchus mykiss*
Furametpyr	4.67 µM	*Cyprinus carpio*
Isofetamid	6.31 µM	*Oncorhynchus mykiss*
Isoflucypram	0.17 µM	*Oncorhynchus mykiss*
Isopyrazam	0.17 µM	*Cyprinus carpio*
Mepronil	37.13 µM	*Oncorhynchus mykiss*
Oxycarboxin	74.44 µM	*Oncorhynchus mykiss*
Penflufen	0.32 µM	*Cyprinus carpio*
Penthiopyrad	0.81 µM	*Pimephales promelas*
	1.07 µM	*Oncorhynchus mykiss*

SDHIs	96 h LC_{50}	Species
	1.59 µM	*Cyprinus carpio*
Pydiflumetofen [15][16] [17] [18] [19] [20] [21] [22], and [23]	0.42 µM	*Oncorhyncus mykiss*
Sedaxane	3.48 µM	*Oncorhynchus mykiss*
	1.96 µM	*Cyprinus carpio*
Thifluzamide	2.46 µM	*Lepomis macrochirus*

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h LC_{50} 2.12 µM (15) and 2.7 µM (16)) (**Table 1** and **Table 2**). However, further work is needed to determine whether these differences are due to species-specific or stage-specific toxicities of the SDHI bixafen.

Because the sensitivity to toxicants may vary according to the stage of individuals, the 96 h LC_{50} values of thifluzamide [23], flutolanil [18], and fluxapyroxad [19] were also determined for zebrafish adults and larvae, and Qian et al. [21] also studied the acute toxicity of penthiopyrad in zebrafish larvae (**Table 1**).

4. Developmental Toxicity of SDHI Fungicides

Besides the acute toxicity of SDHIs and their LC_{50} values for embryos, larvae, or adults, we know that environmental toxicants can also induce adverse effects impairing various developmental processes. Zebrafish embryos have provided versatile tools to characterize the developmental toxicities of the nine SDHIs reviewed here and also help in deciphering their associated adverse outcome pathways. In particular, because the mode of action of SDHIs is inhibition of SDH/CII in fungi, zebrafish embryos have been instrumental in investigating the effects of these fungicides on mitochondrion metabolism, fatty acid synthesis, and reactive oxygen species (ROS) accumulation. The adverse effects of these SDHIs on the development and functioning of the CNS, and on behavior, are reviewed below.

Embryos exposed to bixafen at 0.9 µM for 48 h showed decreased hatching rate and developmental abnormalities, including tail shortening, spinal curvature, and pericardiac edema [15][16]. In addition, exposure to bixafen at 0.3 µM and above caused markedly decreased pigmentation of the trunk and retina [15][16].

5. Long-Term Toxicity of SDHI Fungicides

The determination of the acute toxicity concentrations inducing adverse effects and the associated modes of action is essential for estimating the dangerousness of pesticides and setting appropriate regulations. However, the characterization of the adverse effects induced following long-term exposure to low doses of any pesticide is much

more relevant to the situations encountered in natural environments, and adult zebrafish have been used as tool to evaluate the adverse effects caused by long-term (14–60 days) exposure to low doses of the SDHIs boscalid, flutolanil, and thifluzamide.

The long-term toxicity of boscalid to adult zebrafish was first studied by Qian et al. [24]. The results showed that exposure to boscalid at 0.29 µM for 28 days caused a decrease in weight and length, blood glucose content, hexokinase and SDH activities, and triglyceride content, and an increase in glycogen content in the liver. In individuals exposed to 0.029 µM, a decrease in the activity of fatty acid synthase (FAS) and acetyl coenzyme A carboxylase (ACC), combined with increased expression of the gene encoding G6Pase, was also observed. Lastly, gene expression analysis also confirmed that boscalid at 0.29 µM induced downregulation of *fas* and other genes involved in lipid metabolism, such as *srebp1*, *mgst1*, and *hmgcra*. More recently, Qian et al. [25] observed that the diameters of the adult eye and cornea, together with the photoreceptor layer, were significantly decreased following 21-day exposure to boscalid at 0.29 µM and above.

6. Neurotoxicity and Behavior Deficits Induced by SDHI Fungicides

All animals, including fish, need a fully differentiated and functional central nervous system to find food, escape predators, reach adulthood and sexual maturity, and, ultimately, have offspring. Consequently, any neurotoxicant impairing neuron proliferation, axon pathfinding, synapse formation, axon myelination, neurotransmission, or any other process required for brain functioning can be detrimental to a species in the wild. However, it has long been known that the central nervous system is especially sensitive to toxic insults [26]. In particular, owing to the essential requirement for aerobic energy metabolism in the proper functioning of brain neurons, these cells constitute a likely target for pesticides whose mode of action relies on the inhibition of the mitochondrion ETC, such as SDHI fungicides. In addition, during brain development, a large number of finely regulated processes take place in the absence of a fully functional blood–brain barrier, making the developing brain particularly susceptible to neurotoxicants [26]. However, as highlighted below, few studies have so far investigated the neurotoxicity of SDHI fungicides and especially their adverse effects on neurodevelopment and behavior following low-dose long-term exposure.

The neurotoxicity of bixafen was first investigated by Li et al. [15]. They showed that the expression levels of the *neuroD* and *crx* and *sox2* genes linked to early neurogenesis were significantly downregulated after exposure to 0.3 and 0.9 µM bixafen, respectively, while *nkx2.4b* was upregulated (0.9 µM). In addition, downregulation of genes encoding proteins involved in cell cycle processes was observed in embryos exposed to 0.9 µM bixafen, suggesting that microcephaly of zebrafish embryos was at least partially caused by cell cycle inhibition. We also showed [16] that exposure to bixafen at 0.2 and 0.5 µM for 96 h induced dose-dependently reduced locomotion of embryos, likely the result of defective innervation of body muscles by motoneuron axons, which failed to properly innervate trunk muscles. The data confirmed that exposure to bixafen 0.2 and 0.5 µM also caused microcephaly.

The adverse effects of boscalid on CNS development and functioning were investigated in two recent studies. First, Wang et al. [27] showed that embryos exposed for 48 h to boscalid at 14.56 µM and above displayed gross brain defects, including decreased number of newborn neurons, enlarged brain ventricles, and reduced number of spontaneous movements. In addition, 6 dpf larvae exposed for 24 h to boscalid at 14.56 µM displayed markedly decreased locomotion. Using environmentally relevant concentrations, Qian et al. [25] found that larvae exposed for 7 days to boscalid at 0.87 and 1.74 µM showed significant inhibition of locomotor abilities and reduced phototactic response, respectively. Following 4 or 8 days of exposure to boscalid at 1.74 µM, larvae also showed decreased AChE activity and defects in cerebellar granule cell and retina neuron differentiation. Long-term toxicity studies (21 days) of boscalid toward adults indicated that exposure to 2.9 µM caused significant inhibition in average velocity and acceleration, but a significant increase in active time and distance moved, and exposure to 0.029 µM markedly impaired predatory abilities. Lastly, transcriptome analysis indicated changes in the expression of genes related to neurodevelopment in embryos exposed to bixafen at 1.74 µM for 96 h or 0.87 µM for 8 days, with downregulation of *mbp* and *synapsinIIa*, and upregulation of *gap43*. In addition, several genes required for eye development and phototransduction, *opn1sw1*, *opn1mw1*, *opn4.1*, and *rho,* were significantly upregulated following exposure to 0.87 µM boscalid for 8 days but downregulated with higher concentrations (3.49 µM). Exposure to subacute doses of boscalid thus impaired several essential neuro-behavioral processes, locomotion, and the ability to detect prey, possibly caused by visual system defects and a severe reduction in cerebellar granule cells.

The neurotoxicity of flutolanil toward embryos was investigated by Yang et al. [18]. The results first showed that genes involved in the circadian rhythm were significantly downregulated in embryos exposed for 96 h to flutolanil at 0.38 µM and above. The data also indicated that dopamine content was markedly increased (1.54 µM), the number of spontaneous movements was decreased (0.38 µM and above), and the expression of the *mao*, *th*, and *dbh* genes, encoding proteins involved in neurotransmitter synthesis, was significantly decreased (0.38 and 6.19 µM). Yang et al. [18] also showed that flutolanil at 0.38 µM markedly decreased the number of spontaneous movements of embryos and the expression of many genes encoding both positive and negative regulators of circadian rhythm: *clock1a*, *bmal1a*, *bmal1b*, *bmal2*, *aanat2*, *per1b*, *per2*, *per3*, *cry1aa*, *cry1ab*, *cry1ba*, and *cry1bb*.

The adverse effects of penthiopyrad on behavior were described by Qian et al. [21]. The data showed that embryos exposed to penthiopyrad at 0.83 µM for 8 days showed markedly reduced swimming velocity, acceleration speed, distance moved, and inactive time.

Yao et al. [24] investigated the adverse effects induced by sedaxane on embryos and observed microcephaly in individuals exposed to 6.35 µM and above for 5 days. However, further studies are needed to characterize the adverse outcome pathways involved.

Yang et al. [28] first observed that embryos exposed to thifluzamide 3.6 µM for 96 h displayed severe brain morphology defects. In particular, a marked reduction in the number of neurons was detected in the optic tectum and cerebellum. Also, Yang et al. [28] found that following 96 h exposure to thifluzamide at 3.6 µM, embryos displayed a dramatic decrease in dopamine content and major changes in the expression of genes involved in circadian rhythm, with increased expression of *clock1a*, *per1a*, *per1b*, *per2*, *per3*, *cry1aa*, *cry1ab*, *cry1ba*, *cry1bb,*

cry2, and *cry3*. Thifluzamide at 0.36 µM also caused upregulation not only of *clock2*, *bmal1a*, *balm2*, *aanat2 per2*, *cry1ba*, and *cry1bb*, but also of *mao* and *dbh*, involved in neurotransmitter synthesis. These data show that thifluzamide, like flutolanil, may cause disruption of circadian rhythms, which are essential for proper fish behavior in wild environments.

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