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Effects of the azole fungicide Imazalil on the development of the ascidian *Ciona intestinalis* (Chordata, Tunicata): Morphological and molecular characterization of the induced phenotype

Giuliana Zega, Fiorenza De Bernardi, Silvia Groppelli, Roberta Pennati*

Dipartimento di Biologia, Università di Milano, Via Celoria, 26, I-20133 Milano, Italy

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ABSTRACT

Imazalil (IMA) is a fungicide that is used extensively in fruit plantations and post-harvest treatments, but has teratogenic effects on vertebrate development, possibly due to the perturbation of retinoic acid (RA) levels in the embryo. Ascidians are sessile marine invertebrate chordates that develop through a tadpole larva, with a body plan that shares basic homologies with vertebrates. In this work, we tested the effects of IMA on the development of the solitary ascidian *Ciona intestinalis* by treating two-cell stage embryos with a range of concentrations (0.1, 0.5, 1, 2.5, 5, 10, 20 and 50 μM). The fungicide significantly altered ascidian development even at low concentrations and its effects were dose-dependent. Probit analysis revealed that the median lethal concentration, LC_{50}, was 4.87 μM and the median teratogenic concentration, TC_{50}, was 0.73 μM. Larvae developing from embryos exposed to IMA showed malformations of the anterior structures, which became more severe as IMA concentration increased. In particular, the anterior nervous system and the sensory vesicle were reduced, and the pigmented organs (the ocellus and the otolith) progressively lost their pigmentation.

The larval phenotype induced by 5 μM IMA exposure was further characterized by means of molecular analysis, through whole mount *in situ* hybridization with probes for genes related to the nervous system: *Ci-Otp*, *Ci-GAD*, *Ci-POU IV*, which are markers of the anterior neuro-ectoderm, the central nervous system and the peripheral nervous system respectively, and *Ci-Hox-1*, a gene specifically activated by RA, and *Ci-Aldh2*, a gene for aldehyde dehydrogenase, which is involved in RA synthesis. The altered expression of *Ci-Otp*, *Ci-GAD*, *Ci-POU IV* in 5 μM IMA-exposed larvae compared to control larvae showed that this fungicide could affect the differentiation of the anterior nervous system, particularly of the sensory vesicle neurons. Recent studies suggest a similarity between IMA- and RA-induced phenotypes in tunicates, indicating that triazoles may also alter RA metabolism in ascidians. The observed *Ci-Hox-1* and *Ci-Aldh2* expression in control and treated larvae did not allow a direct link between IMA teratogenic potential and RA-dependent morphogenesis to be identified. It is likely that the fungicidal teratogenic mechanism involved RA signalling but that its effects on ascidian development depend on a more complex mechanism.

1. Introduction

Pesticides used in agriculture represent a risk for aquatic ecosystems. In fact, their uncontrolled use determines the accumulation of these toxic compounds in rivers and coastal habitats, whose communities could be strongly endangered (Castillo et al., 2006). Imazalil (IMA: 1-[2-(2,4-dichlorophenyl)-2(2-propenyloxy)ethyl]-1H-imidazole) is a fungicide, belonging to the group of imidazole and triazole-derivatives, that inhibits fungal cell wall synthesis by interfering with a specific cytochrome P450 enzyme. IMA is used extensively in fruit plantations and post-harvest treatments (Ortelli et al., 2005). It degrades in soils at a rate of 9% in about 4 months and is stable in aqueous solutions for up to 2 months, thus it can easily accumulate in waters, soils and sediments (FAO, 2001). Recently, IMA was found at a concentration of 1 μg/L in waters surrounding a banana plantation, where the composition of macro-invertebrate communities was significantly altered compared to that of reference sites (Castillo et al., 2006).

The antifungal triazoles have teratogenic effects both on vertebrate and invertebrate embryos. In vitro exposure of rat embryos to triazoles (from 100 to 1000 μM) caused branchial arch malformations, involving skeletal, mesenchymal and nervous portions. In particular, the first and second branchial arches were reduced and

* Corresponding author. Tel.: +39 0250314765; fax: +39 0250314802.
E-mail address: roberta.pennati@unimi.it (R. Pennati).

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others were fused (Di Renzo et al., 2007; Menegola et al., 2006, 2004, 2003). Similarly, branchial apparatus development was severely affected by triazoles in the frog *Xenopus leavis* (Groppelli et al., 2005).

In vertebrates, triazole-derivatives inhibit CYP26 enzymes (cytochromes) by interfering with retinoic acid (RA) catabolism (Di Renzo et al., 2007; Van Den Bossche et al., 1989). RA is an important morphogen that regulates gene expression and differentiation by means of a gradient of concentration along the antero-posterior axis. Perturbation of this gradient during early development leads to typical malformations of the anterior structures (Ross et al., 2000).

Ascidians (Chordata, Tunicata) are marine filter feeders. They develop through a planktonic larva, which is formed by a trunk, bearing attachment sensory organs, adhesive papillae or palps, and a tail (Burighel and Cloney, 1997). This larva possesses a hollow dorsal neural tube divided into four main regions: the sensory vesicle, which encompasses a group of photoreceptor cells surrounding the ocellus, the otolith and main nerve centers, located in the posterior portion; the neck; the visceral ganglion, which contains neurons with ascending projections and motor neurons that innervate the tail muscles; and the nerve cord, formed by ependymal cells only (Imai and Meinertzhagen, 2007).

In previous studies, we tested the effects of azoles on the development of the solitary ascidian *Phallusia mammillata*. *P. mammillata* embryos exposed to azole-derivatives showed characteristic malformations of the palp region, the sensory vesicle and the associated nervous network (Groppelli et al., 2007; Pennati et al., 2006). The specific alteration of anterior structures suggested that the teratogenic action of azoles in ascidians also depends on perturbation of the RA pathway.

Ciona intestinalis is a cosmopolitan species that has become the most studied solitary ascidian, following release of its complete genome by Dehal et al. (2002). The aim of this work was to test the teratogenic potential of IMA on *C. intestinalis* development and to characterize the induced phenotype by means of morphological and molecular analysis. For this latter purpose, the expression pattern of some marker genes was studied by means of whole mount *in situ* hybridization, in larvae that developed from IMA-exposed embryos. In particular, we analyzed the expression pattern of three genes related to nervous system differentiation: *Ci-Otp*, a transcription factor that is a specific marker of the anterior neuro-ectoderm (Moret et al., 2005); *Ci-GAD*, the GABA synthesis enzyme, glutamic acid decarboxylase, which is widely expressed in the larval central nervous system (Zega et al., 2008); and *Ci-POU IV*, a transcription factor that is specifically expressed in neurons of the peripheral nervous system (Candiani et al., 2005). Moreover, to evaluate the possible implication of RA in the teratogenic mechanism of IMA, we also studied the expression pattern of *Ci-Hox-1*, a developmental regulatory gene specifically activated by RA, and *Ci-Aldh2*, a gene for aldehyde dehydrogenase, which is involved in RA synthesis (Nagatomo and Fujiwara, 2003).

2. Materials and methods

2.1. Animals and treatments

Adults of *C. intestinalis* were collected in Roscoff, France, and reared in aquaria. Gametes were used for *in vitro* fertilization. Fertilized eggs were allowed to develop in 9-cm petri dishes in Millipore-filtered seawater (mesh size 0.45 μm) at 18 °C. One hour after fertilization, two-cell stage embryos were collected for treatment with Imazalil (IMA) (FW = 297.15, Sigma, Italy). Embryos were exposed to different concentrations of IMA (0.1, 0.5, 1, 2.5, 5, 10, 20 and 50 μM), throughout development (16 h post-fertilization, hpf), without renewing the solution. For each treatment, three replicates were performed, each one using 30 ± 3 embryos, for a total of about 90 specimens for each exposure concentration. Each treatment was replicated in different egg batches, on different dates. Hatched larvae from controls and exposed embryos were fixed with a few drops of formalin and observed and counted under a dissection microscope, to score normal, malformed (shortened trunk and tail, small sensory vesicle, loss of pigment of sensory organs) and undeveloped specimens (dead before completing development). After that, some larvae for each treatment were mounted on glass slides, with 80% glycerol, and photographed with an optical microscope equipped with a Leica DFC 320 digital camera, using the IM50 Leica program.

2.2. Probe synthesis and in situ hybridization

Bluescript SK(+/−) plasmids containing, as inserts, the coding sequences of *Ci-Hox-1* and *Ci-Aldh2* genes were provided by Nagatomo and Fujiwara (2003). Plasmids containing *Ci-POU IV* and *Ci-GAD* coding sequences were available in our lab (Candiani et al., 2005; Zega et al., 2008). The *Ci-Otp* clone was amplified from *C. intestinalis* cDNA using PCR, with the following primers—F: 5′-ATTGTGGCAAACAACAACGA-3′; R: 5′-TTCGCTTCTTCCATTTTGCT-3′(Moret et al., 2005), and inserted in a pCR-II topo vector (Invitrogen, Italy). The identity of clones was systematically checked by sequencing.

RNA probes of marker genes were synthesized, after DNA extraction and linearization, from bacterial clones transformed with the different plasmids, using T7 and Sp6 polymerase, following the instructions supplied with the DIG RNA labeling kit (Roche Diagnostics SpA, Italy). These probes were then used in whole-mount *in situ* hybridization (ISH).

Pre-hatching (14 hpf) and hatched larva (16 hpf) of controls and 5 μM IMA-exposed embryos were first fixed in fresh 4% paraformaldehyde in 0.5 M NaCl and 0.1 M MOPS at pH 7.5, at room temperature for 90 min. Then, samples were dehydrated in an ethanol series, and stored at −20 °C in 70% ethanol. ISH was then performed according to the protocol described in Gionti et al. (1998). Probes encoding for regulatory developmental genes (*Ci-Otp*, *Ci-POU IV*, *Ci-Hox-1*) were used for ISH in pre-hatching larvae, as they are generally highly expressed during embryonic development. *Ci-Aldh2* expression was studied in both pre-hatching embryos and hatched larvae. *Ci-GAD* expression was determined only in hatched larvae, as this gene is a marker for differentiated GABA-ergic neurons. The probes were detected using an anti-DIG antibody, conjugated with an alkaline phosphatase, and revealed by color reaction, with NBT/BCIP substrates. For each probe, at least 30 treated specimens were processed and analyzed.

All labeled embryos and larvae were mounted on glass slides with 80% glycerol, and were observed using an optical microscope. One specimen, randomly chosen, was photographed for each probe. All IMA-exposed embryos and larvae showed typical gene expression patterns.

For histological analysis, samples were dehydrated, counterstained with 1% red Ponceau in 1% acetic acid, embedded in Spurr resin, and sectioned at 3 μm.

All images were acquired with a Leica DFC 320 digital camera, using the IM50 Leica program.

2.3. Statistical analysis

Probit analysis was performed to estimate median lethal and median teratogenic concentrations (LC_{50} and TC_{50}), in order to calculate the teratogenic index of IMA (TI) (Finney, 1971).

Analysis of variance, followed by HSD Tukey's post hoc test, was used to evaluate differences in the teratogenic effects of the series of IMA concentrations. Percentage data were arcsine transformed to