Mechanistic Approaches to Screening Chemicals for Endocrine Toxicity Using an Invertebrate

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Science Question

The currently proposed Tier 1 screening battery for detecting endocrine activity of chemicals suffers from two major deficiencies. 1) No invertebrate screen is included despite the proposed use of a Tier 2 multigenerational test with a crustacean. 2) The battery is not equipped to assess combined effects of diverse endocrine toxicants. The overall objectives of this proposed research program is to develop a mechanism-based high-throughput screening assay for evaluating estrogen, androgen, and thyroid (EAT)-like activities in an invertebrate species that also can be used to evaluate interactive effects of endocrine-active compounds through receptor cross-talk.

Research Goals

The first goal of this research program is to identify endpoints that could be applied to a high-throughput screening format to detect endocrine toxicity of chemicals using a whole-organism invertebrate model. The crustacean *Daphnia magna* is being used as the test organism for this purpose since: 1) it is commonly-used in toxicity testing, 2) its endocrinology has been well characterized, 3) it is more amenable to high-throughput testing than the recommended species for Tier 2 testing (mysid shrimp), and 4) results obtained will be applicable to mysids and other arthropods. EAT-like activities in crustaceans are under the control of ecdysteroids and terpenoids. These endocrine signaling pathways are the primary focus of this program. Cross-talk between signaling pathways provides a mechanism by which diverse endocrine toxicants can interact resulting in greater than additive or unanticipated toxicity. The second goal of the program is to define mechanisms of cross-talk between hormone signaling pathways and use this information to refine the screening assay with appropriate apical and molecular endpoints. This research will provide the foundation for the development and use of a screening assay that is diagnostic of endocrine toxicity to arthropods and can be used to screen chemicals mixtures for occult endocrine toxicity.

Approach/Results

I. Identify apical endpoints of endocrine activity that could be applied to a high-throughput screening format.

Ia. Distinct developmental abnormalities are associated with anti-ecdysteroidal activity of chemicals.

For example, the agricultural fungicide fenarimol reduced ecdysteroid levels in embryos which caused distinct developmental abnormalities. These abnormalities could be prevented by providing ecdysteroid to the embryos.

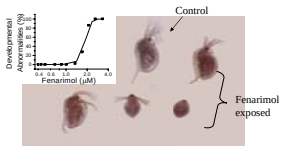


Fig. 1 Developmental abnormalities among embryonic daphnids exposed to fenarimol. All organisms depicted are of the same age.

Ib. Chemicals with terpenoid hormone activity caused male sex determination among embryos when exposed during ovarian oocyte maturation.

For example, the insecticide pyriproxyfen mimicked the action of the terpenoid hormone methyl farnesoate with ~300-fold greater potency. Maternal daphnids exposed to 0.10 μg/l pyriproxyfen produce ***only*** male offspring.

Fig. 2 Female and male daphnids. The male was produced artificially as a result of in ovo exposure to the insecticide pyriproxyfen.

Ic. Exposure to low levels of terpenoid mimics, in combination with elevated temperature, causes bilateral gynandromorphic offspring.

For example, exposure of maternal daphnids to pyriproxyfen at 30ºC resulted in offspring that exhibited female characteristics on one side of the body and male characteristics on the other. We hypothesize that elevated temperature induces HSP proteins that overly stabilize the terpenoid receptor and interfere with its signaling. Experiments are underway to determine whether some chemicals can similarly induce HSPs resulting in a synergistic effects between these chemicals and terpenoid mimics.

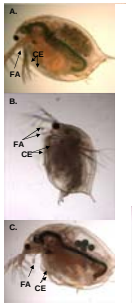


Fig. 3 Female (A), male (B), and gonandromorphic (C) daphnids. First antennae (FA) are diminutive in females and elongated in males. Gynandromorphs have one short and one long antennae. The carapace edges (CE) of females are symmetrical and are asymmetrical in males. Gynandromorphs have one symmetrical and one asymmetrical carapace edge.

Id. Chemicals with terpenoid hormone activity induce hemoglobin

Fig. 4 Color change (hemoglobin induction) in response to exposure to pyriproxyfen. A. untreated, B. pyriproxyfen treated.

Fig. 5 Screening protocol for assessing endocrine activity of chemicals.

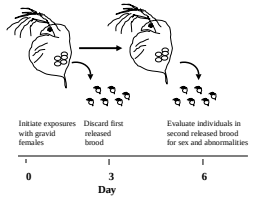


Table 1 Results of screening assays for endocrine activity using the apical endpoints described.

Chemical	Concentration (μg/l)	Terpenoid activity	Terpenoid potentiation	Anti-ecdysteroid activity
control	-	-		-
methyl farnesoate	200	+		-
juvenile hormone III	20	+		+
pyriproxyfen	0.10	+		+
fenoxycarb	1.0	+		+
methoprene	310	+	+	-
kinoprene	1000	-	+	-
retinoic acid	800	-	+	-
phytanic acid	300	-		-
fenarimol	1300	-		+
azadirachtin	1.0	-		-
tert-amylphenol	1000	-		-
dieldrin	100	+		-
cis-chlordane	10	-		-
piperonyl butoxide	300	-		+
bisphenol A	10000	-	+	+
4-nonylphenol	200	-		+
sodimum nitroprusside	140	-		+
ammonium perchlorate	1000	-		+
testosterone	3000	-		+

IIA. Mechanisms of endocrine interaction that have been evaluated to date.

IIA1. Terpenoid potentiation. Weak (methoprene) or inactive (kinoprene) terpenoids, retinoic acid, and bisphenol A have all been shown to potentiate the activity of administered methyl farnesoate (Table 1). These compounds might competitively-inhibit terpenoid esterases resulting in elevated concentrations of the active hormone (methyl farnesoate).

IIA2. Synergy among hormone synthesis inhibitors and receptor antagonists. Fenarimol inhibits ecdysteroid synthesis. Testosterone antagonizes the ecdysteroid receptor (EcR). Together, fenarimol reduced endogenous levels of ecdysteroids increasing the competitive advantage of testosterone for binding to and antagonizing the EcR resulting in greater that additive endocrine toxicity.

IIA3. Antiecdysteroidal activity of terpenoids. Terpenoids elicit antiecdysteroidal activity but do not antagonize the EcR or reduce ecdysteroid levels. We propose that terpenoids bind RXR which is the heterodimeric partner to the EcR (see Fig 6) and prevent activation of the EcR:RXR transcription factor. Combined activity of a terpenoid mimic and a classical antiecdysteroid may be greater than additive.

IIB. Novel mechanisms of endocrine disruption under Investigation.

IIB1. Antiecdysteroidal and antiterpenoid activity of nitric oxide generators (sodium nitroprusside, nitrite, nitrate). We propose that nitric oxide can bind to the signaling protein E75 (NR1D3) which then negatively regulates both ecdysteroid and terpenoid signaling (Fig. 6 model, Fig. 7 & 8 supporting data). E: ecdysteroid; EcR: ecdysteroid receptor; MF: methyl farnesoate.

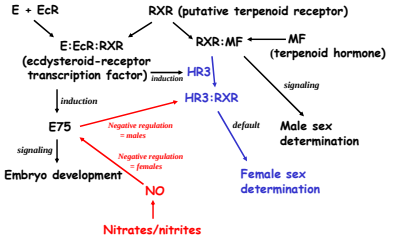


Fig. 6 Proposed ecdysteroid/terpenoid signaling web and its disruption by nitrates/nitrites.

Fig. 7 Antiecdysteroid-like activity of nitric oxides.

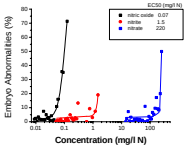
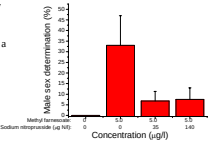


Fig. 8 Antijuvenoid-like activity of sodium nitroprusside (nitric oxide generator).



Impact and Outcomes

- Several apical endpoints have been identified and characterized that are indicative of endocrine toxicity in a crustacean.
- A 6-day screening assay was developed using these endpoints.
- Molecular endpoints have been tentatively identified that could complement the apical endpoints, particularly in identifying interactions among endocrine-active compounds.

Future Directions

- Identify regulators of hormone signaling (i.e. EcR, RXR, HR3, E75 that are modulated (induced/suppressed) in response to endocrine-active compounds.
- Clone and sequence these cDNAs.
- Generate probes to these regulators that can be used to monitor endocrine activity of chemicals in a high-throughput screening format.
- Establish whether RXR is the methyl farnesoate receptor of crustaceans.
- If not, then determine the identity of this important signaling molecule.
- Definitively establish the roles of E75 and HR3 in endocrine signaling.
- Definitively establish the ability of nitrates/nitrites to interfere with normal endocrine signaling.

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Long Term Goal II

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