Tributyltin Synergizes with 20-Hydroxyecdysone to Produce Endocrine Toxicity

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One of the great challenges facing modern toxicology is in predicting the hazard associated with chemical mixtures. The development of effective means of predicting the toxicity of chemical mixtures requires an understanding of how chemicals interact to produce nonadditive outcomes (e.g., synergy). We hypothesized that tributyltin would elicit toxicity in daphnids (Daphnia magna) by exaggerating physiological responses to 20-hydroxyecdysone signaling via synergistic activation of the retinoid X receptor (RXR):ecdysteroid receptor (EcR) complex. Using reporter gene assays, we demonstrated that RXR, alone, is activated by a variety of ligands including tributyltin, whereas RXR:EcR heterodimers were not activated by tributyltin. However, tributyltin, in combination with the daphnid EcR ligand 20-hydroxyecdysone, caused concentration-dependent, synergistic activation of the RXR:EcR reporter. Electrophoretic mobility shift assays revealed that tributyltin did not enhance the activity of 20-hydroxyecdysone by increasing binding of the receptor complex to a DR-4 DNA-binding site. Exposure of daphnids to elevated concentrations of 20-hydroxyecdysone caused premature and incomplete ecdysis resulting in death. Tributyltin exaggerated this effect of exogenous 20-hydroxyecdysone. Further, exposure of daphnids to tributyltin enhanced the inductive effects of 20-hydroxyecdysone on expression of the 20-hydroxyecdysone-inducible gene HR3. Continuous, prolonged exposure of maternal daphnids to concentrations of tributyltin resulted in mortality concurrent with molting. Taken together, these results demonstrate that xenobiotics, such as tributyltin, can interact with RXR to influence gene expression regulated by the heterodimeric partner to RXR. The result of such interactions can be toxicity due to inappropriate or exaggerated hormonal signaling. The application of the in vitro/in vivo approach used in this study is discussed in relation to modeling of nonadditive interactions among constituents of chemical mixtures.

Key Words: chemical mixtures; models; synergy; invertebrate; ecdysteroid; nuclear receptor; retinoid X receptor; ecdysteroid receptor

Hormone nuclear receptors are well recognized as targets of toxicity associated with endocrine-active chemicals (Iguchi and Katsu, 2008). Nuclear receptors are designed to bind small lipophilic molecules, and once bound, these molecules modify the ability of the nuclear receptor to regulate gene transcription either positively (as an agonist) or negatively (as an antagonist). Most nuclear receptors function as regulators of gene expression as dimers and several nuclear receptors form heterodimeric combinations with the retinoid X receptor (RXR) (Chawla et al., 2001). Such heterodimeric receptors offer two distinct ligand-binding sites with which chemicals (endogenous or exogenous) can interact to modify receptor function.

Vertebrate RXR heterodimers have been categorized as permissive or nonpermissive (Shulman and Mangelsdorf, 2005). Permissive heterodimers are subject to activation by ligands to either receptor partner. Occupancy of both partners by their cognate ligands can result in synergistic activation of the receptor (Li et al., 2002; Mu et al., 2000). Example, permissive partners to RXR include the peroxisome proliferator–activated receptor (PPAR), the liver X receptor (LXR) and the farnesoate X receptor (FXR). Among nonpermissive heterodimers, ligand binding to RXR does not activate the complex.

The arthropod ecdysteroid receptor (EcR) functions as a prototypical heterodimeric transcription factor with RXR (Yao et al., 1993). In response to ecdysteroid hormones that bind within the ligand-binding domain of the EcR, the RXR:EcR complex regulates a variety of activities related to development, growth, and reproduction (LeBlanc, 2006). RXR is typically considered to be a non–ligand-binding silent partner in this arrangement (Iwema et al., 2007). However, Maki et al. (2004) reported that juvenile hormone III elicited a suppressive action on ecdysteroid activation of the Drosophila RXR:EcR in a reporter gene assay system. Conversely, we observed that the crustacean sesquieterpenoid hormone, methyl farnesoate, enhanced ecdysteroid activation of the daphnid RXR:EcR (Wang and LeBlanc, 2009). Studies with the Drosophila RXR ortholog (termed USP) have shown that methyl farnesoate binds to the receptor with significantly greater affinity than does juvenile hormone III (Jones et al., 2006).
Virtually no consideration has been given to the potential for environmental chemicals to modify the activity of endogenous hormones through cooperative activity at nuclear receptor heterodimers resulting in endocrine toxicity. Furthermore, dual binding sites on nuclear receptor heterodimers provide a potential target for combined toxicity of binary mixtures of environmental chemicals. These interactions may provide a mechanism of synergy among environmental chemicals that warrants investigation.

Considering the precedent for synergistic interactions between endogenous RXR ligands and the endogenous ligands of the partner receptor to RXR, we hypothesized that RXR heterodimers may provide a molecular target for synergistic interactions between xenobiotics or between xenobiotics and endogenous hormones resulting in endocrine-related toxicity. We tested this hypothesis by evaluating synergistic interactions between the biocide tributyltin, the first documented activator of an arthropod RXR (Wang and LeBlanc, 2009) and the EcR ligand 20-hydroxyecdysone at the molecular level. Whole-organism experiments involving the water flea *Daphnia magna* were used to anchor these observed molecular interactions to perturbations in molting, an ecdysteroid-regulated process.

** MATERIALS AND METHODS **

**Chemicals.** 20-Hydroxyecdysone, tributyltin chloride, triphenyltin chloride, and 9-cis retinoic acid were purchased from Sigma-Aldrich and all were ≥ 95% pure. LGD1069 (> 99% purity) was purchased from LC Laboratories (Woburn, MA). LG100268 was provided by Dr Seth Kullman, Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, NC. LGD1069 and LG100268 are synthetic retinoids that are potent activators of mammalian RXR (Boehn et al., 1995). Chemicals were dissolved in absolute ethanol for daphnid exposures at a final concentration of 0.0015%. Chemicals were delivered to media used in reporter assays dissolved in dimethyl sulfoxide at a final concentration of 0.05%.

**Animal exposures.** Daphnids (*Daphnia magna*) used in this study were obtained from cultures maintained in our laboratory for over 18 years. Daphnids were reared in media reconstituted from deionized water as described previously (Wang et al., 2007). Cultured daphnids were maintained at a density of 40 adults per 1000 ml of media and were fed twice daily with 2.0 ml (1.4 × 10^6 cells of algal and 0.10 mg (dry wet) of fish food 7 days old. Experiments were maintained in incubators as described previously (Hannas and LeBlanc, 2010).

Whole-animal exposures were performed with animals that were of the same age (as indicated in individual experiments) and molt synchronized. Molt synchronized animals were generated as described previously (Hannas and LeBlanc, 2010). Animals were individually treated in 50-ml beakers containing 40 ml of culture media. Solutions were exchanged every 2–3 days. Test beakers were provided 3.5 × 10^6 cells of algal and 0.10 mg (dry wet) of fish food homogenate twice daily for daphnids ≤ 7 days old and twice these amounts, for animals > 7 days old. Experiments were maintained in incubators as described for cultures.

**Electrophoretic mobility shift assays.** Electrophoretic mobility shift assays (EMSAs) were used to assess binding of daphnid RXR and EcR on the DR-4 oligonucleotide probe. The biotin-labeled DR-4 probe (Durica et al., 2002) consisted of the oligonucleotides 5′-TTGGACAAGGTCACAG-GAGGTCACTTCTTT/3biotin-TEG-3′ (IDT, Coralville, IA). The underlined nucleotides denote the direct repeat (DR) half-sites which are separated by four nucleotides. *Drosophila* S2 cells were propagated as described for reporter gene assays. Cells were seeded at a density of 3 × 10^5 in a 35-mm plate and transfected 16–23 h after plating. Transfections were performed by calcium phosphate DNA precipitation with 4 μg of daphnid RXR or daphnid EcR. Following transfection, cells were washed and transcription induced with the addition of CuSO₄ at a final concentration of 500μM for 24 h.

Cells were harvested and the nuclear proteins were extracted using commercially available reagents (Pierce, Rockford, IL). One microliter of nuclear extract from both the daphnid RXR and the daphnid EcR-producing cells were incubated with 2 μl of 10× binding buffer (Pierce), 1 μl of 1 μg/μl of poly dI-dC, 20 fmol of biotin-labeled DR-4 probe, and ultrapure water to a total volume of 20 μl. Competition assays also contained 4 pmol of unlabeled DR-4 probe (IDT). The reaction was incubated at room temperature for 20 min, 5 μl of 5× loading buffer was added to each reaction and the entire sample (25 μl) was loaded and subjected to electrophoresis in a 6% DNA retardation gel (Invitrogen, Carlsbad, CA) at 100 V for 60 min. After electrophoresis, the proteins were transferred to Biodyne B nylon membrane (Pierce) at 80 V for 40 min in 0.5× trisborate/EDTA buffer. Protein and DNA were cross-linked to the membrane with UV light. Biotin-labeled DNAs were detected by chemiluminescence using commercially available reagents (Pierce).

**Luciferase reporter gene assays.** Construction and cloning of the constituents of the reporter gene assays (GAL4-RXR(DEF), GAL4-RXR(DEFp)-Flag-EcR(ORG)) were described previously (Wang and LeBlanc, 2009). Reporter gene assays were performed in *Drosophila* Schneider (S2) or HepG2 cells, as indicated. *Drosophila* S2 cells were propagated in Schneider’s medium (Gibco, Carlsbad, CA), containing 10% heat-inactivated fetal bovine serum (Gibco), 50 units/ml penicillin G (Fisher Scientific, Pittsburgh, PA), 50 μg/ml streptomycin sulfate (Fisher Scientific) and incubated at 23°C under ambient air atmosphere. HepG2 cells were grown in Mediatech Cellgro minimum essential medium (Fisher Scientific) containing 10% fetal bovine serum and incubated at 37°C in a 5% CO₂ atmosphere. Cells were transfected when at 50–70% confluence. Transfections were performed using TransIT-LT1 Transfection Reagent (Mirus, Madison, WI), according to the manufacturer’s protocol. Each well received 0.5–1.0 μg of the appropriate receptor-containing vectors along with 0.10 ng vector containing pSV-β-galactosidase which served as a control for transfection efficiency and pG5Luc which contained the luciferase gene under the control of the GAL4 response element. Cells used to assess transcriptional activation by the daphnid GAL4-RXR(DEF) receptor also were transfected with 1.0 μg of the coactivator SRC-1 (Wang and LeBlanc, 2009). Transfected cells were treated with the putative ligands for 24 h and harvested for luciferase determinations. Luciferase activities were measured using the Luciferase Assay System (Promega, Madison, WI) and normalized to β-galactosidase activities which were measured using the β-galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega), according to the manufacturer’s protocols. Each experiment was repeated at least three times.
The membrane was incubated in blocking buffer for 15 min and then in 10 ml of blocking buffer containing 33.4 μl streptavidin-HRP solution for 15 min. The membrane was washed with 1× washing buffer four times and then placed in substrate equilibration buffer for 5 min. The membrane was incubated in a mixture consisting of 1 ml of luminol/enhancer solution and 1 ml peroxide solution for 2 min and then exposed to X-ray film for 2–5 min.

**Polymerase chain reactions.** Whole-adult female daphnids were homogenized with a dounce homogenizer and RNA was isolated using the SV Total RNA Isolation System (Promega). RNA yield was determined by absorbance at 260 nm and its purity was measured by the 260/280 nm absorbance ratio. RNA integrity was verified by formaldehyde agarose gel electrophoresis. RNA was reverse transcribed to cDNA with oligo dT primers using the ImProm-II Reverse Transcription System (Promega).

Relative expression of the daphnid HR3 gene was determined by quantifying gene transcript levels using quantitative real-time qRT-PCR. Daphnid HR3 oligonucleotide primer sequences used were described previously (Hannas and LeBlanc, 2010). Actin (accession #AJ292554) and GAPDH (accession #AJ292555) cDNA were also amplified and used in the normalization of transcripts (Hannas et al., 2010). Quantitative real-time PCR was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using default parameters. Amplification mixtures consisted of 12.5 ml SYBR Green PCR Master Mix (Applied Biosystems), 300nM primers and 20 ng template cDNA in a total volume of 25 μl. Primer concentrations were optimized following the manufacturer’s recommendations. The reaction mixtures were first kept at 95°C for 5 min, followed by 40 cycles with each cycle consisting of a temperature of 95°C for 5 s followed by 60°C for 1 min. After the PCR reactions, the melting temperature of PCR product was determined using the dissociation protocol provided by the instrument manufacturer. A single melting peak was detected for all samples indicating no amplification of nontarget DNA. Furthermore, only a single amplification product was detected following electrophoresis in a 1% agarose gel and staining with ethidium bromide. The comparative Ct (CT) method (2−ΔΔCT) was used to assess the relative levels of messenger RNA (mRNA). Validation experiments, as described by the instrument manufacturer, confirmed that the efficiencies of the target and endogenous controls (actin and GAPDH) amplifications were approximately equal.

**Statistics.** Significant differences among several means were assessed using ANOVA and Dunnet multiple comparison test. Equality of variances and normality were established using Levene test and the Shapiro-Wilk test, respectively. Quantal data were analyzed using Fisher exact test with significance set at a probability of ≤ 0.05.

**RESULTS**

**Receptor Activation**

Initial experiments were performed to definitively characterize the ligand activation of the daphnid RXR in a luciferase-based reporter assay. No activation of the reporter was observed at concentrations as high as 1.0μM of the vertebrate RXR ligands 9-cis retinoic acid and LGD1069, along with the crustacean sesquiterpenoid hormone and putative RXR ligand (Jones et al., 2006) methyl farnesoate (Fig. 1). The reporter was activated by the ligands LG100268, triphenyltin, and tributyltin in a concentration-dependent manner. Among these ligands, tributyltin was the most potent activator of RXR. Accordingly, all subsequent experiments were performed using tributyltin as the RXR ligand.

Next, the modifying effect of tributyltin on 20-hydroxyecdysone-mediated activation of the ecdysteroid receptor complex (daphnid RXR:EcR) was evaluated. Consistent with previous observations (Wang and LeBlanc, 2009), a concentration of 0.52μM 20-hydroxyecdysone provided minimal activation of the luciferase gene in a reporter assay (Fig. 2A). Increasing concentrations of tributyltin progressively enhanced reporter activation above that observed with 20-hydroxyecdysone alone (Fig. 2A). Tributyltin, alone, had no effect of reporter activity (Fig. 2B). Taken together, these results indicate that when daphnid RXR is in heterodimeric confirmation with daphnid EcR, the RXR ligand has no effect on receptor activation (i.e., nonpermissive). However, binding of tributyltin to daphnid RXR, when associated with daphnid ecdysteroid-bound EcR, results in a synergistic activation of the receptor complex. The lack of reporter activation by tributyltin alone also indicates that, in this system, the heterodimeric association between daphnid RXR and EcR is favored over the RXR homodimer, which is activated by tributyltin.

The synergistic activity between 20-hydroxyecdysone and tributyltin may result from increased binding to their DNA response element when both components of the receptor complex are associated with ligand. Electrophoretic mobility shift assays were performed to test this possibility. Daphnid EcR alone and daphnid RXR alone exhibited no discernible binding to a DR-4 oligonucleotide probe (Fig. 3A, lanes 1–4), the prototypical RXR:EcR response element (Durica et al., 2002; Elke et al., 1999). However, when added together, a significant shift in the DR-4 probe occurred (Fig. 3A, lanes 5,6). This shift represented specific binding to the DR-4 probe as inclusion of 100-fold excess unlabelled probe resulted in the loss of the band (Fig. 3A, lane 7). Inclusion of EcR ligand,
20-hydroxyecdysone, resulted in enhanced binding to the DR-4 probe (Fig. 3A, lanes 8 and 9). These results demonstrate that the daphnid RXR:EcR heterodimer specifically binds to the DR-4 sequence, and this binding is enhanced by the presence of EcR ligand. Inclusion of tributyltin along with the daphnid RXR, daphnid EcR, and 20-hydroxyecdysone resulted in no enhanced binding to the DR-4 probe beyond that observed with 20-hydroxyecdysone alone (Fig. 3B). In conclusion, EMSA results demonstrate that tributyltin does not synergize with 20-hydroxyecdysone by increasing binding affinity of the receptor complex to the DR-4 response element.

**In Vivo Synergy**

Among the ecdyzoans, the steroid hormone 20-hydroxyecdysone orchestrates the molting process (ecdysis) in coordination with peptide hormones such as eclosion hormone (Gammie and Truman, 1999). Exposure of daphnids to exogenous 20-hydroxyecdysone resulted in a significant occurrence of incomplete ecdysis (Fig. 4). Exposure of daphnids to 20-hydroxyecdysone concentrations as high as 0.73 μM or tributyltin concentrations as high as 3.8 nM caused no significant increase in the occurrence of incomplete ecdysis.
incomplete ecdysis (Fig. 5). However, coexposure of daphnids to various combinations of 20-hydroxyecdysone and tributyltin did significantly increase the occurrence of incomplete ecdysis (Fig. 5). Thus, tributyltin synergized with 20-hydroxyecdysone to produce toxicity consistent with elevated levels of 20-hydroxyecdysone alone.

**Gene Expression**

HR3 is an ecdysteroid-inducible gene in daphnids (Hannas and LeBlanc, 2010) and other ecdyzoans (Jindra and Riddiford, 1996; Palli *et al.*, 1995). Experiments were performed to determine whether the synergistic action between 20-hydroxyecdysone and tributyltin also was evident at the level of *in vivo* gene expression. Exposure of daphnids to 1.0µM 20-hydroxyecdysone resulted in a < 2-fold increase in HR3 mRNA levels (*p* = 0.06) (Fig. 6). Exposure to 3.8nM tributyltin had no effect on HR3 mRNA levels. However, in combination, these treatments significantly (*p* = 0.0035) elevated HR3 mRNA levels. Consistent with the synergistic effects of 20-hydroxyecdysone and tributyltin on *in vitro* gene expression and *in vivo* exuviation, coexposure to these materials also synergized at the level of *in vivo* gene expression.

**Endocrine Toxicity of Tributyltin**

All *in vivo* experiments described thus far were performed with exogenous administration of 20-hydroxyecdysone. The following experiment was performed only with tributyltin to...
determine whether this biocide would elicit toxicity consistent with synergism with endogenous 20-hydroxyecdysone. Neonatal daphnids (< 24 h old) were exposed to a series of concentrations of tributyltin for 14 days during which time survival, exuviation, and reproduction was evaluated. Daphnids exposed to tributyltin concentrations /C20 3.3nM survived and reproduced during the 14-day experiment (Fig. 7). On average, these daphnids produced more offspring than was observed among control organisms (control: 20 ± 9, mean ± SD, n = 10) and exhibited no discernible concentration-related trend in reproductive output (Fig. 7B). Daphnids exposed to tributyltin concentrations ranging from 3.6 to 5.0nM perished on the 12th day of the experiment concomitant with exuviation and release of the first brood of offspring (Fig. 7A). Daphnids exposed to tributyltin concentrations greater than 5.0nM died during a juvenile exuviation. These results demonstrate that tributyltin elicits toxicity characterized by lethality at the time of exuviation when 20-hydroxyecdysone levels are at their highest (Martin-Creuzburg et al., 2007; Mu and LeBlanc, 2004). Unlike experiments where organisms were exposed to exogenous 20-hydroxyecdysone, lethality was not associated with incomplete ecdisis.

**DISCUSSION**

The arthropod RXR had often been considered to be a ligand-independent transcription factor (Tocchini-Valentini et al., 2009) until we demonstrated that the daphnid RXR was activated by tributyltin (Wang and LeBlanc, 2009). In the present study, we expand the breadth of ligands that activate daphnid RXR to include triphenyltin and LG100268. Although clearly ligand activated, an endogenous ligand to the daphnid RXR remains elusive since neither the prototypical vertebrate RXR ligand 9-cis retinoic acid (Heyman et al., 1992) nor the RXR-binding arthropod hormone methyl farnesoate (Jones et al., 2006) activates the receptor in a reporter assay.

We also demonstrate in the present study that while daphnid RXR alone functions as a ligand-activated transcription factor, its ligand is incapable of activating the daphnid RXR:EcR receptor complex. Rather, ligand-bound RXR modifies the action of 20-hydroxyecdysone-ligated EcR. Specifically, tributyltin functions synergistically to enhance activation of the RXR:EcR receptor complex by 20-hydroxyecdysone both in vitro and in vivo. This synergy caused toxicity when 20-hydroxyecdysone was exogenously provided to the daphnids and elicited toxicity, in the absence of exogenous 20-hydroxyecdysone, that was consistent with synergy between the organotin and endogenous hormone. These interactions define a mechanism by which chemicals may synergize to produce endocrine toxicity. Specifically, low, nontoxic concentrations of an EcR agonist may interact with an RXR ligand to cause aberrant ecdysteroid signaling with resulting toxicity. Consequences of such endocrine disruption would include impaired ecdisis, as observed in the present study, and suppressed vitellogenesis which is negatively regulated by ecdysteroids in some arthropods (Hannas et al., 2010).

EcR is one of many nuclear receptors that forms heterodimeric transactivation complexes with RXR. Others, that are of significance to vertebrates, include TR, PPAR, LXR, FXR, CAR VDR, and PXR (Mangelsdorf and Evans, 1995). These receptor complexes can be functionally categorized as being
with the EcR ligand. This activity is reminiscent of synergistic silent in this arrangement but can bind ligand and synergize typical nonpermissive receptor complexes, daphnid RXR is not only by a ligand to the EcR component. However, unlike as nonpermissive since, in the present study, it was activated result in synergistic activation (Li et al., 2002; Mu et al., 2000). The daphnid RXR:EcR receptor complex is best categorized as nonpermissive since, in the present study, it was activated only by a ligand to the EcR component. However, unlike typical nonpermissive receptor complexes, daphnid RXR is not silent in this arrangement but can bind ligand and synergize with the EcR ligand. This activity is reminiscent of synergistic interactions observed between RXR and RAR in vertebrates (Chen et al., 1996; Taneja and Chambon, 1995).

The target of tributyltin-induced synergism is identified in the present study. However, the mechanism through which this synergism occurs remains elusive. Durica et al. (2002) reported that provision of both RXR and EcR from the fiddler crab (Uca pugilator) was required to bind the DR-4 probe in electrophoretic mobility shift assays and that inclusion of 20-hydroxyecdysone increased the binding to the probe. In the present study, we demonstrate the requirement for both daphnid receptors to bind the DR-4 and increased binding with the provision of ligand 20-hydroxyecdysone. We hypothesized that tributyltin would further increase binding activity of the receptor complex to the DR-4 element. This hypothesis was not supported by electrophoretic mobility shift assays. Perhaps, tributyltin synergizes with 20-hydroxyecdysone by enhancing the recruitment of coactivators to the complex (Wiebel et al., 1999) or by increasing interaction with another response element (Elke et al., 1999).

Modeling of the toxicity of chemical mixtures involving nonadditive interactions among the mixture constituents is in its infancy, despite the obvious need to recognize, predict and regulate chemicals in an effort to mitigate such toxicity. Most modeling efforts currently are nonquantitative, retrospective analyses that are designed simply to recognize the presence of synergy or antagonism (Gennings, 2010; Wilbur et al., 2004). Existing methods for prospective modeling of mixtures toxicity involving nonadditive interactions are extremely data intensive, requiring the quantitative characterization of interactions among mixture constituents for use in the model (Rider et al., 2005). These characterizations typically involve the use of in vivo toxicity assessments performed at various doses of a binary mixture (Rider et al., 2005). This intense data requirement is largely responsible for the limited incorporation of synergistic/antagonistic interactions for the predictive modeling of mixtures toxicity.

The identification of specific targets by which chemicals can interact to produce nonadditive toxicity and the use of these targets in in vitro assessments of interaction provides an alternative means of quantifying interactions for use in mixtures modeling. Such an approach would reduce the cost, time, and animal usage associated with data acquisition and is consistent with recommendations in the National Research Council’s Toxicity Testing in the 21st Century: A Vision and a Strategy (National Research Council, 2007). The use of transcriptional reporter assays, electrophoretic mobility shift assays, cell-based toxicity assessments, and other approaches all could add to the toolbox of approaches for assessing toxicological interactions among chemicals.

Whole-animal assessments of mixtures toxicity provide a viable approach to validating predictive models of mixtures toxicity. The genome of Daphnia pulex was recently deciphered (Colbourne et al., 2011) and was found to surpass humans in the number of genes. Included in the daphnid

![Figure 7](https://academic.oup.com/toxsci/article-abstract/123/1/71/1646202)
genome is a full array of chemical biotransformation enzymes (Baldwin et al., 2009) and nuclear receptors (Thomson et al., 2009). The use of small invertebrate models such as Daphnia sp., Drosophila melanogaster, and Caenorhabditis elegans provide the complexity of the whole organism with significant savings in time, cost, and vertebrate animal investment. Daphnia has recently joined the ranks as an NIH-recognized invertebrate model for biomedical research along with D. melanogaster and C. elegans (http://www.nih.gov/science/models/daphnia/). Future investments in mixtures toxicity assessment should include the continued identification of mechanisms of nonadditive interactions, creation of chemical interaction data sets that would allow for the generation of quantitative metrics for use in modeling assessments, and evaluations of the strengths and limitations of using data derived from in vitro or invertebrate modeling approaches to quantify interactions in vertebrates. The use of nonvertebrate alternative approaches may prove to be the only viable route to assessing the toxicity of complex chemical mixtures.

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