Research Article

Theme: Pharmaceuticals and Personal Care Products in the Environment

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Comparative Pharmacology and Toxicology of Pharmaceuticals in the Environment: Diphenhydramine Protection of Diazinon Toxicity in *Danio rerio* but Not *Daphnia magna*

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Abstract. Pharmaceuticals and other contaminants of emerging concern present unique challenges to environmental risk assessment and management. Fortunately, mammalian pharmacology and toxicology safety data are more readily available for pharmaceuticals than other environmental contaminants. Identifying approaches to read-across such pharmaceutical safety information to non-target species represents a major research need to assess environmental hazards. Here, we tested a biological read-across hypothesis from emergency medicine with common aquatic invertebrate and vertebrate models. In mammals, the antihistamine diphenhydramine (DPH) confers protection from poisoning by acetylcholinesterase inhibition because DPH blocks the acetylcholine receptor. We employed standardized toxicity methods to examine individual and mixture toxicity of DPH and the acetylcholinesterase inhibitor diazinon (DZN) in *Daphnia magna* (an invertebrate) and *Danio rerio* (zebrafish, a vertebrate). Though the standardized Fish Embryo Toxicity method evaluates early life stage toxicity of zebrafish (0–3 days post fertilization, dpf), we further evaluated DPH, DZN, and their equipotent mixture during three development stages (0–3, 3–6, 7–10 dpf) in zebrafish embryos. Independent action and concentration addition mixture models and fish plasma modeling were used to assist interpretation of mixture toxicity experiments. Though our primary hypothesis was not confirmed in acute studies with *Daphnia magna*, DPH conferred a protective effect for acute DZN toxicity to zebrafish when DPH plasma levels were expected to be greater than mammalian therapeutic, but lower than acutely lethal, internal doses. We further observed that timing of developmental exposure influenced the magnitude of DZN and DPH toxicity to zebrafish, which suggests that future zebrafish toxicity studies with pharmaceuticals and pesticides should examine exposure during developmental stages.

KEY WORDS: alternative toxicity test methods; biological read-across; comparative pharmacology and toxicology; mixture toxicology; pharmaceuticals in the environment.

INTRODUCTION

Pharmaceuticals and other chemicals of emerging concern (CECs) present unique challenges to environmental risk assessment (ERA) and risk management (1). Many of the scientific methodologies (e.g., model organisms, computational models) used to assess environmental risks of pharmaceuticals were derived from studies of historical contaminants such as persistent organic pollutants (e.g., polychlorinated biphenyls), heavy metals, and pesticides, which have primarily been

examined for acute toxicity to aquatic life (2). Pharmaceuticals are designed to be biologically active and interact with therapeutic targets at low concentrations; thus, with the exception of developing countries with lower treatment capacity and fewer enforced water quality regulations, sublethal responses of aquatic and terrestrial organisms are more likely to be observed in the environment than acute toxicity (3). Because these sublethal effects are poorly understood in wildlife, a number of timely research needs were recently identified to understand risks of pharmaceuticals in the environment (4).

Assessing risks of historical environmental contaminants is routinely hampered by limited toxicity information. Unlike these historical contaminants, a large repository of mammalian safety information exists for pharmaceuticals (5). In the USA, the Food and Drug Administration examines potential pharmaceutical impacts in the environment under the National Environmental Policy Act (6,7). To begin to address the existing data gaps for pharmaceutical impacts in the environment, comparative pharmacology and toxicology approaches may be developed through “read-across”

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processes (8). Because conservation of critical molecular receptors for pharmaceuticals exist across aquatic vertebrates (9–11), it appears possible to utilize such information from target organisms (e.g., human, mammal) to prioritize chemicals for further study (12,13) and support more robust ERA and environmental decisions (14–16). To organize mammalian data for read-across to aquatic species, an adverse outcome pathway (AOP) (17) provides a critical structural framework for understanding therapeutic mechanism of action (MOA)-specific responses; employing AOPs was recently recommended for future studies examining environmental impacts of pharmaceuticals (18). Further, a recent horizon scanning exercise collected over 400 research questions necessary to better understand risks of pharmaceuticals and personal care products in the environment (4). From these questions, a workshop of global experts identified 20 priority research questions (4). Among the top six research needs identified during this expert workshop were as follows: “How can pharmaceutical preclinical and clinical information be used to assess the potential for adverse environmental impacts of pharmaceuticals?” “What can be learned about the evolutionary conservation of PPCP targets across species and life stages in the context of potential adverse outcomes and effects?”; and “How can effects from long-term exposure to PPCP mixtures on non-target organisms be assessed?” Such research needs prompted the present study.

In the environment, pharmaceuticals with various MOA are observed in complex mixtures (19), particularly in effluent-dominated ecosystems (20). Unfortunately, interactive effects of pharmaceuticals in wildlife are poorly understood. In an effort to understand effects of similar and dissimilarly acting compounds, Backhaus and Faust (21) proposed a two-stage strategy first using concentration addition (CA) modeling and then applying an independent action (IA) model to the CA-predicted response. In both predictive methods, it would be ideal to know or at least anticipate MOAs for compounds in a mixture to develop research hypotheses *a priori*. Here again, employing the AOP framework and comparative pharmacology approaches should provide a basis for interpreting environmentally important mixtures.

In the present study, we explore the use of comparative pharmacology and an AOP framework to interpret interactive pharmaceutical toxicity using two common aquatic animal models. Though the current emergency medicine treatment for organophosphate insecticide (OP) poisoning in mammals is atropine, diphenhydramine (DPH), the active ingredient in Benadryl® and several other human medicines, is considered as an alternative treatment because DPH competitively inhibits the acetylcholine (ACh) receptor, and thus protects against OP poisoning caused by hyperstimulation from acetylcholinesterase inhibition (22–24). Thus, the overarching objective of this study was to test a read-across hypothesis for DPH protection against diazinon (DZN; a model OP) toxicity in the aquatic invertebrate, *Daphnia magna*, and the vertebrate, *Danio rerio*. Based on emergency medicine observations in mammals and conservation of ACh and acetylcholinesterase (AChE) across animal models, we specifically tested the hypothesis that DPH would reduce acute and chronic DZN toxicity. We further examined whether timing of exposure during different developmental

stages influenced the magnitude of DZN, DPH, and mixture toxicity to *Danio rerio*.

METHODS AND MATERIALS

Chemicals and Analytical Verification of Treatment Levels

Diphenhydramine hydrochloride (CAS 147-24-0; ≥98% purity; Sigma-Aldrich Corp., St. Louis, MO, USA) and DZN (CAS 333-41-5; >98% purity; Chem Service, Inc., West Chester, PA, USA) were purchased at the highest purities available. For analytical verification of stock solutions, isotopically labeled DPH-d3 (Cerilliant, Round Rock, TX) and DZN-d10 (Toronto Research Chemicals Inc., Ontario, Canada) were also acquired.

Treatment levels of DPH and DZN from each toxicity study were analytically verified using isotope dilution liquid chromatography tandem mass spectroscopy (LC-MS/MS) with electrospray ionization (ESI). A 500-μL aliquot of undiluted or diluted exposure water samples was combined with 450 μL of 0.1% formic acid (*w/w*) and spiked with 50 μL of an internal standard mixture (DPH-d₃ and DZN-d₁₀) in an autosampler vial before analyses. Instrumentation and strategies for separation and detection of target analytes were primarily described elsewhere (25,26). A gradient mobile phase condition resulted in elution of DPH at 4 min and DZN at 8 min. The ionization mode and monitored transitions for DZN and internal standard DZN-d₁₀, which had not been reported previously by our group, were as follows: ESI+DZN 305.0>169.0 and DZN-d₁₀ 315.0>170.0. The collision energy for DZN and DZN-d₁₀ were −20.0 and −22.5 V, respectively.

Ten standards, ranging in concentration from 0.1 to 500 ng/mL, were utilized to build a linear calibration curve ($r^2=0.998$). Continuing calibration verification (CCV) samples were also run between every five samples to monitor instrument calibration. The majority of analytically verified concentrations (74%) fell within 20% of their nominal values; however, measured concentrations of DPH and DZN ranged from 39.04 to 149.6% of nominal concentrations. For this reason, analytically verified concentrations were used in statistical analyses and to develop figures and tables.

Daphnia magna Studies

Daphnia magna were obtained from permanent cultures and maintained at 25°C with a 16-h light/8-h dark photoperiod. Reconstituted hard water (RHW) (27) was used to maintain cultures and also served as controls for toxicity studies with *Daphnia magna*. RHW water quality parameters included the following: pH 8.5 (±0.1), conductivity 607.4 (±47) μS/cm, hardness 170 (±5.3) mg/L as CaCO₃, alkalinity 106.8 (±8.3) mg/L as CaCO₃, dissolved oxygen 8.3 (±1.2) mg/L (28). Cultures were fed once daily with green algae (*Pseudokirchneriella subcapitata*) and Cerophyll (Cereal Grass Media, Innovating Science™, Aldon Corp, Avon, NY, USA) mixture (27).

Daphnia magna 48-h mortality studies followed standard US EPA protocols (27). Briefly, acute toxicity studies utilized neonates (<24 h old) with five individuals per experimental unit and five replicates per treatment level. Independent acute toxicity studies were performed in triplicate. Neonates

were exposed for 48 h following this experimental design for DPH, DZN, or DZN × DPH mixtures. DPH preliminary studies were performed to confirm treatment levels previously reported by our group (26); the acute DPH EC₅₀ value used for mixture calculations, further described below, was 374 µg/L (26). Treatment level solutions for toxicity studies were prepared with RHW, and for DZN and DZN × DPH mixture studies, methanol was used as a solvent carrier. Solvent controls were included in each study, wherein methanol was equivalent and did not exceed 0.01%. Analytical verification by LC-MS/MS of stock solutions and treatment levels occurred at study initiation and conclusion.

Subchronic toxicity studies with *Daphnia magna* were then performed, following standard 10-day static renewal US EPA protocols (29) with minor modifications previously reported by our group (30). Briefly, this experimental design utilized <24-h-old neonates with one organism per test chamber and 10 replicates of each treatment level. Duplicate studies were performed for DPH, DZN, or DZN × DPH mixtures. During each experiment, organisms were observed and fed daily with a *P. subcapitata* and Cerophyll mixture (31). Treatment level solutions were renewed every other day with stock solutions, which were prepared at test initiation and stored at 4°C. Similar to acute studies, methanol was used as a solvent carrier for DZN and mixture studies and did not exceed 0.01%. Water samples were collected on days 0, 5, and 10 for analytical confirmation of treatment levels by LC-MS/MS.

Danio rerio Studies

Wild-type tropical 5D zebrafish (*Danio rerio*) were kept under standard culture conditions at Baylor University and followed Institutional Animal Care and Use Committee-approved protocols. Adult zebrafish were cultured in a filtered (Compact system, Lifeguard Aquatics, Cerritos, CA, USA) recirculating system (Marine Biotech Systems, Beverly, MA, USA) maintained at 28°C on a 16-h light/8-h dark photoperiod and housed in 1 L aquaria with densities of ≤5–6 fish per tank. System culture water was formulated by addition of Instant Ocean® salts (Aquatic Eco-Systems, Inc, Apopka, FL, USA) (60 mg/L) to deionized water; the media was brought to pH 7 with sodium bicarbonate (50 mg/L). Culture water quality parameters included the following: pH 6.7 (±0.1), conductivity 565 (±114) µS, and dissolved oxygen 7.8 (±0.6) mg/L. Zebrafish were fed twice daily with brine shrimp (*Artemia nauplii*; Aquatic Eco-Systems, Inc, Apopka, FL, USA) and once daily with AES flake fish food (Aquatic Eco-Systems, Inc, Apopka, FL, USA).

Though standardized experimental designs for zebrafish embryos are initiated shortly after fertilization (e.g., OECD test no. 236 (32)), in the present study, acute toxicity of DPH, DZN, or their binary mixture to zebrafish was evaluated for three developmental periods. The three 72-h exposure windows selected were 0–3, 3–6, and 7–10 days post fertilization (dpf). Following staging described by Kimmel *et al* (33), experiments were initiated between the shield and 75% epiboly stages (6–8 hours post fertilization, hpf) or at days 3 and 7, respectively. All studies were renewed and observed daily. Stock solutions of DPH, DZN, or their mixture were prepared with zebrafish culture water (Instant Ocean® in DI water) at study initiation and were stored at 4°C in the dark. For DZN and mixture treatment levels, methanol was

used as a solvent carrier, but did not exceed 0.01%. Here again, solvent controls were included for DZN and mixture studies. Water samples of stock solutions were taken for analytical verification by LC-MS/MS concentration quantification.

For the 0–3 dpf experiments, ten 6–8 hpf embryos were placed in 10 mL of solution in a 30-mL chamber, with 20 chambers per treatment level. For both the 3–6 and 7–10 dpf exposures, 10 larvae were placed in 80 mL of solution in 100-mL beakers, with five beakers per concentration. Range finding tests were conducted to initially identify concentrations for study. Embryos or larvae were exposed for 72 h to DPH, DZN, or DPH × DZN mixtures. Water samples were collected at study initiation for analytical confirmation of treatment levels by LC-MS/MS.

Mixture Models

Mixture treatment levels were formulated utilizing single chemical toxicity data to calculate mixture units (S units; see individual chemical toxicological benchmark concentrations in Table I), where $1S = 0.5(EC \text{ or } LC_{50DZN}) + 0.5(EC \text{ or } LC_{50DPH})$. A dilution series was created from the 1S formulation, with two concentrations above and below this central value (e.g., 4S, 2S, 1S, 0.5S, and 0.25S). To evaluate chemical mixture interactions, the CA and IA models were evaluated; predicted values were calculated for each model and compared to observed mixture toxicity data. The CA model predicts the expected combined effect of similarly acting agents (34) via the summation of the fractions of individual constituents in a given mixture. For example, in the case of a two component mixture, the predicted concentration of the two chemicals (described as a toxic unit (TU) with an S value) that elicits X% effect (e.g., of an LC₅₀) is determined by the relationships of the concentrations of these two substances to their respective individual toxicological benchmark values (e.g., LC₅₀), where C_a and C_b are the concentrations of the substances in the mixture as expressed in Eq. 1.

$$TU = \frac{C_a}{LC_{50a}} + \frac{C_b}{LC_{50b}} \quad (1)$$

Conversely, the IA model predicts the interaction of dissimilarly acting toxicants (34,35) and was first proposed by Bliss in 1939 (Eq. 2):

$$R_m = R_a + R_b - (R_a \times R_b) \quad (2)$$

where R_m = response to the mixture of chemicals a and b , and R_a or R_b = response to the individual chemicals (35).

Statistical Analysis

Acute EC or LC₅₀ values were calculated with the US EPA Toxstat software using the Probit method if data met statistical assumptions; however, if they did not meet Probit assumptions then the trimmed Spearman-Kärber method was used (27; Table I). For 10-day reproductive studies, no observable effect concentrations (NOECs) and lowest observable effect concentrations (LOECs) were identified using analysis of variance with Dunnett's post-hoc test ($\alpha=0.05$; (36,37)).

Table I. Statistical Analyses Used to Estimate EC₅₀ and LC₅₀ Values for *Daphnia magna* and *Danio rerio*, Respectively, Following Exposure to Diphenhydramine, Diazinon, or their Equipotent Mixtures

Organism	Age window	Diphenhydramine	Diazinon	Mixture
<i>Daphnia magna</i>	NA	–	TSK	TSK
<i>Danio rerio</i>	0–3 dpf	Probit	TSK	Probit
	3–6 dpf	Probit	TSK	TSK
	7–10 dpf	TSK	TSK	TSK

TSK trimmed Spearman-Kärber, dpf days post fertilization

RESULTS

Daphnia magna Studies

Table II lists the analytically verified acute 48-h EC₅₀ values and subchronic 10-day NOEC and LOEC values for *Daphnia magna* toxicity studies. Concentration-dependent relationships were consistently observed for *Daphnia magna* mortality and fecundity. In both survival and reproduction, DZN was more potent than DPH. For example, an order of magnitude difference was observed between NOECs and LOECs for DZN and DPH (Table II). However, DZN exhibited a much smaller margin between acute and subchronic responses with little difference between the EC₅₀ (0.896 µg/L) and NOEC (0.35 µg/L; Table II). Conversely, the NOEC and EC₅₀ values for DPH differed by 2 orders of magnitude. In the case of 10-day survival, significant ($p < 0.05$) responses were seen in the highest DPH and two highest DZN concentrations (Fig. 1a). For DZN, reductions in neonate production per female were noted to only be significant when significant reductions in survival were also observed (Fig. 1a, b). However, this was not the case for DPH because significant reproduction effects were evident at treatment levels not resulting in significant survival responses.

Following studies to examine equipotent mixtures of DPH and DZN, *D. magna* responses were plotted against predicted concentration addition and independent action model values (Figs. 2 and 3). For acute mixture studies, toxicity was under predicted by CA below 1S, but then appeared to more closely align with predicted CA values at 1,

2, and 4S for mortality (Fig. 2). Conversely, for the subchronic studies, at low mixture treatment levels, both the CA and IA models overpredicted toxicity. At higher (2S, 4S) concentrations resulting in significant fecundity responses (Fig. 3a), the CA model appeared to more closely describe interactive effects on sublethal responses (Fig. 3b).

Danio rerio Studies

Analytically verified 72-h toxicity thresholds for DPH and DZN are listed in Table II. Similar to observations with *Daphnia magna*, DZN was more potent than DPH for the different embryonic and larval stages (Table II). During each exposure window examined, LC₅₀ values were an order of magnitude lower for DZN than for DPH (Table II). Further, LC₅₀ values for DZN and DPH decreased with increasing age of the fish (Table II; Fig. 4). DZN LC₅₀ values were 14.7, 12.2, and 5.41 mg/L for 0–3, 3–6, and 7–10 dpf studies, respectively. Similarly, for 0–3, 3–6, and 7–10 dpf experiments, LC₅₀ values for DPH were 692, 262, and 45.5 mg/L, respectively.

Following approaches employed for *Daphnia magna* to examine equipotent mixtures of DPH and DZN, *Danio rerio* responses were plotted against predicted concentration addition and independent action model values (Fig. 4). At lower mixture concentrations (0.25S, 0.5S, some 1S), toxicity was overpredicted by both CA and IA models (Fig. 5a–c). For 0–3 and 7–10 dpf at 1S, CA underpredicted acute toxicity. At elevated (2S, 4S) mixture concentrations, empirical data for all three exposure windows were in agreement with CA but not consistently with IA predictions (Fig. 5a–c).

DISCUSSION

Aquatic toxicology of OP insecticides has been extensively examined, particularly for aquatic invertebrates and vertebrates. For example, Williams *et al.* (38) recently identified acetylcholinesterase inhibition (AChEI) hazard concentrations at the 5th centile of probability distributions of 0.188 and 65.07 ng/L for *Daphnia magna* and *Pimephales promelas* (fathead minnow), respectively, which illustrates the potency of these common substances compared to other environmental contaminants. DZN is commonly associated with its use as insecticide, but is also used as a veterinary medicine (39). The primary objective of the present study was

Table II. Analytically Verified EC₅₀ or LC₅₀ Values (mean±SD; $n=3$) and No Observed Effect Concentrations (NOEC) and Lowest Observed Effect Concentrations (LOEC) for Diazinon (DZN) and Diphenhydramine (DPH), and their Equipotent Mixtures (Second Two Rows) in *Daphnia magna* 48-h Acute and 10-Day Subchronic Fecundity Studies, and *Danio rerio* 72-h Acute Studies

		<i>Daphnia magna</i>		<i>Danio rerio</i> 72 h LC ₅₀ (mg/L)		
		Mean 48-h EC ₅₀ (µg/L)	10-day NOEC, LOEC (µg/L)	0–3 dpf	3–6 dpf	7–10 dpf
Individual chemical	DZN	0.896 (±0.1)	0.363, 0.627	14.7	12.2	5.41
	DPH	374 (±142) ^a	2.94, 5.94	692	262	45.5
Mixture	DZN	0.435 (±0.15)	0.42, 0.99	6.89	7.41	3.11
	DPH	205 (±78)	1.78, 3.57	269	159	19.81

Dpf days post fertilization

^a Berninger *et al.* (24)

Fig. 1. *Daphnia magna* subchronic (10 days) **a** survival and **b** fecundity responses to either diphenhydramine or diazinon. Statistical differences from controls are denoted with asterisks (* $p < 0.05$)

to test a biological read-across hypothesis from the emergency medicine literature between mammals and common aquatic model organisms. We specifically hypothesized that DPH would confer a protective effect for DZN poisoning in

Fig. 2. Evaluation of *Daphnia magna* acute toxicity (48 h EC_{50}) for equipotent mixtures of diphenhydramine (DPH) and diazinon (DZN). S values denote equipotent mixtures based on individual chemical respective EC_{50} values ($1S = 0.5(EC_{50DZN}) + 0.5(EC_{50DPH})$)

Fig. 3. **a** Interactive effects of equipotent mixtures of diphenhydramine (DPH) and diazinon (DZN) on mean ($\pm$ SD) *Daphnia magna* fecundity (10 days). **b** Comparison of concentration addition (CA) and independent action (IA) models to mean *Daphnia magna* fecundity (10 days, percent of control) of equipotent mixtures of DPH and DZN. S values denote equipotent mixtures based on individual chemical respective LOEC values ($1S = 0.5(LOEC_{DPH}) + 0.5(LOEC_{DZN})$). Statistical differences from controls are denoted with asterisks (* $p < 0.05$)

aquatic invertebrates and vertebrates. Threshold toxicity values observed here for *Daphnia magna* to DZN generally followed previous reports for both acute (40–42) and subchronic (42,43) endpoints. Though DPH and other antihistamine toxicity data for invertebrates is not as readily available as DZN (44), *Daphnia magna* observations from the present study are similar to previous reports by our group (26) and others (45). Such toxicity threshold observations for DZN or DPH are typically higher than observed in surface waters or effluents in developed countries; however, the objectives of the present study did not include examining mixture response thresholds to environmental exposure scenarios. For example, we recently reported *Daphnia magna* 10-day reproduction NOEC and LOEC values of 0.8 and 3.4 $\mu\text{g/L}$ for DPH (26), which are quite similar to the *Daphnia magna* sublethal response thresholds reported in Table II.

In contrast to our hypothesis based on the mammalian emergency medicine literature, DPH did not uniformly ameliorate DZN acute toxicity in daphnia, particularly at

Fig. 4. Acute (72 h) *Danio rerio* mortality of **a** diazinon (DZN) and **b** diphenhydramine (DPH) during three stages of development (0–3, 3–6, and 7–10 days post fertilization, dpf)

lower treatment levels, and, furthermore, deviations from the CA and IA models were observed (Fig. 2). However, daphnia reproduction responses were more closely predicted by both the IA and CA models (Fig. 3). CA and IA represent the predominate approaches to predict mixture toxicity (21). Though DPH and DZN displayed distinct concentration-response toxicity to *Daphnia magna* and zebrafish (Figs. 1 and 4), which then were used to estimate EC_{50} values and to select corresponding equipotent treatment levels for mixture toxicity studies, this approach does not account for different slopes between concentration-response relationships. Backaus *et al* (46) recently found that formulating mixtures using ratios of EC_{50} values, as was done in the present study, resulted in difficulties in mixture modeling if tested mixture ratios utilized individual component concentrations eliciting <1% effect due to differing slopes for each of the individual chemicals. Subsequently, treatment levels derived from individual EC_{50} values may differ from mixture modeling predictions particularly if these substances were not present at appropriate mixture concentrations and ratios to mechanistically interact as modeled.

In contrast to observations for daphnia acute toxicity, zebrafish mortality more closely aligned with our read-across hypothesis from mammalian emergency medicine at lower (e.g., below 1S) but not higher treatment levels of equipotent mixtures of DZN and DPH (Fig. 5). Because treatment levels in the present study were selected around acute toxicity

Fig. 5. Comparison of concentration addition (CA) and independent action (IA) models compared to observed mortality (72 h LC_{50}) from equipotent mixtures of diphenhydramine (DPH) and diazinon (DZN) in *Danio rerio* for **a** 0–3 dpf; **b** 3–6 dpf; and **c** 7–10 dpf studies. S values denote equipotent mixtures based on individual chemical respective LC_{50} values ($1S = 0.5(LC_{50DZN}) + 0.5(LC_{50DPH})$). dpf = days post fertilization

thresholds, DPH could have exerted toxicity through its multiple therapeutic MOAs or by general toxicity (e.g., narcosis) at these higher concentrations (Fig. 5). In an effort to interpret observations from the present study across the equipotent treatment levels (Fig. 5), we employed a fish

plasma uptake modeling approach (10,26,47) to predict internal doses of DPH of zebrafish in Fig. 5 using Eq. 3:

$$P_{BW} = \left(10^{0.73 \log D (\text{pH } 7)} - 0.16\right) + 0.84 \quad (3)$$

where P_{BW} is the blood/water partition coefficient (47) and $\log D$ represents the octanol/water partition coefficient of DPH (a weak base) based on pH (26) of the zebrafish experiments. If the mammalian DPH $C_{\max}$ value (0.05 $\mu\text{g/mL}$) is used as a surrogate for an internal therapeutic dose in fish (10,16,26), then zebrafish DPH plasma levels would be expected to range from 30 to 7,500 $\times$ of the human therapeutic dose. Bird *et al* (23) identified a protective DPH effect for OP poisoning in rats treated with 30 mg/kg, which corresponds to $\sim 43\times$ higher dosage than required to reach a therapeutic effect (0.7 mg/kg). In contrast, the rat oral LD_{50} value of 390 mg/kg for DPH is $\sim 557\times$ greater than the therapeutic dosage. Thus, at low mixture concentrations where CA and IA models overestimated DPH and DZN toxicity to zebrafish (Fig. 5), internal doses of DPH were likely consistent with internal plasma levels demonstrated by Bird *et al* (23) to be protective of OP poisoning. Similarly, at higher equipotent mixture treatment levels (e.g., greater than 1S; Fig. 5), zebrafish plasma concentrations would have exceeded internal doses associated with rat mortality (e.g., LD_{50} values). Based on observations and experimental designs employed in the present study, the mammalian to aquatic read-across hypothesis tested appears plausible for zebrafish, but not daphnia, likely due to differences in evolutionary conservation pharmaceutical targets in aquatic vertebrates and vertebrates (9,48). Such information appears to present valuable approaches for prioritization pharmaceuticals with higher likelihood to elicit adverse outcomes to non-target organisms (4,49), and thus support more robust environmental research, assessment, and monitoring (14).

Another high priority research need identified by Boxall *et al* (4) was: "How can ecotoxicity test methods that reflect the different modes of action of active PPCPs be developed and implemented in customized risk assessment strategies?" Early life stages are sensitive to contaminant exposure; regulatory toxicology experimental designs were subsequently developed to include early life stages in toxicity testing, particularly for common models such as *P. promelas* (27), *Daphnia magna* (27), and *Danio rerio* (32). However, sensitivity to some toxicants varies during developmental stages (49); for example, it has previously been demonstrated that sensitivities to OPs increase over development in amphibians (50) and fish (51,52).

In the present study, we also observed similar sensitivity shifts; increasing developmental stage exhibited decreases in LC_{50} values for DZN (Table I). This increase in sensitivity following DZN exposure is linked to rational molecular design of second generation OPs insecticides wherein metabolism to a more toxic oxon metabolite is responsible for elevated AChEI and thus toxicity. Biotransformation to the oxon metabolite of DZN occurs by cytochrome (CYP) P450 enzyme oxidation, yet expression and activity of CYP enzyme isoforms fluctuate in intensity and location over development (53–55). Additionally, an understanding of pharmaceutical metabolism in fish species is lacking (15,56,57); although some mammalian homologues have been identified, functional conservation has not yet been established for most CYPs

(56). Despite this uncertainty, DZN acute toxicity differences across development stages observed in the present study correlated with potential bioavailability influences by the presence the chorion, a selectively permeable membrane surrounding the embryo. Although permeability inherently varies across species and during development, it may be that because zebrafish can be chorionated until 72 hpf, lower sensitivity of the 0–3-dpf study relative to the later two development stages resulted from reduced bioavailability of DZN. For example, previous studies in Japanese medaka have attributed differences in sensitivity to decreased uptake during chorionated stages, and increased sensitivity to dechorionated stages due to elevated CYP activity (51,52). Other studies have also noted bioavailability-related effects due to the presence of the chorion (58–60). In fact, Embry *et al* (61) noted the poorly understood influences of the chorion on bioavailability of various chemicals. Clearly, the influence of chorionation on bioavailability and toxicity of industrial chemicals and effluents deserves additional study.

Interestingly, we observed a similar increase in sensitivity with development exposure for DPH studies. Though an increase in sensitivity from the 0–3-dpf study to the 3–6- and 7–10-dpf experiments may have resulted from dechorionation (completed by 3 dpf), a continued decrease in LC_{50} values for DPH (Table I) between the 3–6- and 7–10-dpf experiments was also observed. In humans, DPH is transformed by CYP2D6 to several metabolites, including *n*-desmethyldiphenhydramine (or nordiphenhydramine), which maintains some biological activity (62). Unfortunately, differential mammalian toxicity among these metabolites is not well documented, and information on toxicity of DPH metabolites to zebrafish is lacking. However, CYP 2D6 activity does not appear present in fish models; for example, recent observations by our group indicated that DPH is not transformed *in vitro* by rainbow trout liver S9 (15), which suggests that metabolism may not represent a major contributor to observations of increased toxicity with development observed in the present study.

Though DPH is often associated with allergy treatment, resulting from H1 receptor antagonism, it also exhibits therapeutic activity by blocking serotonergic and cholinergic receptors. Serotonin, histamine, and acetylcholine receptors are present in zebrafish; some of which display varying patterns of expression through development (63,64). For example, serotonin reuptake transporter (SERT) expression is differentially expressed in regions of the zebrafish hypothalamus; SERT expression is not observed until 4 dpf, though serotonin has been detected as early as 2 dpf (65). Thus, it appears that pharmacodynamics or pharmacokinetic differences across developmental stages may have contributed toxicity with zebrafish age in the present study. Such observations indicate that standardized experimental designs for toxicity testing should consider initiating exposure during multiple stages of zebrafish development to reduce uncertainties during chemical hazard and risk assessments.

CONCLUSIONS

Leveraging mammalian pharmacology and toxicology data for pharmaceuticals presents opportunities to more efficiently and robustly assess and manage environmental risks. In the present study, the utility of a biological read-across hypothesis, initially derived from the emergency medicine literature, was

observed in acute toxicity studies with the aquatic vertebrate *Danio rerio*, but not the aquatic invertebrate *Daphnia magna*, likely due to evolutionary conservation of pharmacological targets across vertebrates. We further observed developmental timing of toxicity test initiation to influence the magnitude of toxicity to zebrafish, indicating that future alternative environmental and biomedical studies with zebrafish should examine exposure across developmental stages.

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Conflict of Interest None to declare.

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