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The Assessment of a Six Day Larval Zebrafish Model for Determining Acute Toxicity to Organophosphorus Compounds

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U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC) was previously known as U.S. Army Edgewood Chemical Biological Center (ECBC).

We developed a larval zebrafish acute toxicity screening tool for acetylcholinesterase (AChE)-inhibiting compounds. Our model uses the ability of the six day post-fertilization (DPF) larval zebrafish to produce AChE and various cytochrome P450 (CYP) metabolizing enzymes that activate or detoxify organophosphorus (OP) chemical species to modulate toxicity. We utilized 6 DPF *Danio rerio* embryos to examine nerve agent lethality and AChE inhibition relative to the OP pesticide parathion (PT). We also assessed the more active form of the CYP desulfonated OP, paraoxon (PT-O). We first compared the 24 h lethality response of *D. rerio* embryos to VX with their response to PT and PT-O. VX was observed to be 1000× more potent than PT. We then compared the AChE-inhibiting potential of VX relative to OPs when normalized to the lethal dose. VX induced a 100% inhibition of AChE. The -oxon derivative, PT-O, was notably more toxic than its parent compound. VX demonstrated lethality and AChE inhibition similar to that of active oxon rather than the parent pro-compound. This work demonstrates the ability of the 6 DPF zebrafish to be a competent model for nerve agent potency and measurement of AChE inhibition. These characteristics factor into operational risk assessments for traditional and emerging threat compounds.

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PREFACE

The work described in this report was authorized under Defense Threat Reduction Agency (Fort Belvoir, VA) project number CB10595. The work was started in January 2018 and completed in June 2019. At the time this work was performed, the U.S. Army Combat Capabilities Development Command Chemical Biological Center (DEVCOM CBC; Aberdeen Proving Ground, MD) was known as the U.S. Army Edgewood Chemical Biological Center (ECBC).

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This report has been approved for public release.

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THE ASSESSMENT OF A 6 DAY LARVAL ZEBRAFISH MODEL FOR DETERMINING ACUTE TOXICITY TO ORGANOPHOSPHORUS COMPOUNDS

1. INTRODUCTION

Background

Organophosphorus (OP) compounds are of particular concern to the Department of Defense and the broader community. Because of their widespread use as pesticides in agriculture, they are readily available and accessible in large quantities, thus creating the potential for mass exposure.[1–3] In addition, OP compounds are relatively easy to synthesize, creating the possibility for their use as a chemical threat.[1–3] These characteristics have generated a need to develop tools for the rapid assessment of OP compounds and their associated toxicity, how they affect human health, and the determination of possible countermeasures.[4,5]

Organophosphate compounds are a class of acetylcholinesterase (AChE) inhibitors that include pesticides and nerve agents.[6,7] They interact with AChE through covalent binding and prevent the breakdown of the neurotransmitter acetylcholine (ACh), leading to cholinergic crisis at the synapse.[8,9] Chemical examples of organophosphate pesticides include parathion (PT), malathion (MT), and chlorpyrifos (CP). Representatives of organophosphates include chemical warfare agents (CWAs) such as sarin, tabun, and *o*-ethyl s-diisopropylaminomethyl methylphosphonothiolate (VX).[8] AChE functions to hydrolyze ACh, breaking down the neurotransmitter and preventing its accumulation in the synaptic cleft, thus ultimately regulating the neuronal synaptic transmission and supporting body homeostasis.[9,10] Inhibition of AChE by OP compounds causes ACh to accumulate at cholinergic synaptic clefts.[9,11,12] Failure to metabolize the neurotransmitter leads to the overstimulation of cholinergic neurons, causing a cascade of events including hyper excitation, seizures, and secondary neuronal effects.[6,9,11,12] Although previous studies have determined the depth of organophosphate poisoning and researched remedies to counteract the toxicity of organophosphate compounds to humans, development and validation of rapid screening systems that can assess organophosphate characteristics and initiate potential countermeasures are essential in understanding organophosphates and preparing for potential exposures.

Advancement in developing high-throughput testing methods is critical with millions of compounds used in manufacturing, medicine, and agriculture. Pertaining to OPs and the need for countermeasure determinations, high-throughput screening systems equipped to effectively and efficiently determine the potential impacts of chemicals on human health should be developed and validated. Although in vitro assays are fundamental in high-throughput systems (HTS) that target specific organ toxicity effects of chemicals, in vivo platforms enable HTS to provide toxicity data that encompass the complexity of a whole organism.[4,8,13] A valuable in vivo model should provide data that account for the complexity of human physiology and still be effective in screening tens to thousands of different compounds.[8] The zebrafish, *Danio rerio,* has emerged as a viable model organism for such reasons and has been used for

studies in pharmacology and biomedical research, specifically in large-scale chemical screening studies.[8,11,14,15] The zebrafish was used in toxicological research because of its genetic similarity to humans, small size, fecundity, low cost, and relevance to the physiological complexity of mammalian model systems.[4,6,8,15–17]Analysis of the zebrafish genome established that 71% of human proteins and 82% of disease-causing human proteins have zebrafish orthologs.[18]

Although rodents have been used to understand relevant pathophysiological mechanisms of OPs and potential treatments, zebrafish have emerged as a viable research organism that allows for high-throughput screening with these compounds.[7,19] In addition, zebrafish AChE catalytic properties and genetic expression are well characterized.[18] They only express the AChE gene, and their AChE enzymes have 62% amino acid sequence similarity with key acyl- and choline-binding center amino acids being conserved between zebrafish and humans.[18,19] Based on their human-like expression of AChE, we aimed to develop an acute toxicity larval screening tool for AChE-inhibiting compounds. Using six day post-fertilization (DPF) zebrafish embryos, we examined nerve agent lethality and cholinesterase inhibition relative to the well-known OP pesticide PT. We also assessed the more active form of the cytochrome P450 (CYP) desulfonated OP, paraoxon (PT-O), and the CWA VX. The ultimate goal of this work was to develop a high-throughput model for screening AChE-inhibiting compounds that can adequately and rapidly assess the toxic effects of OPs, which could potentially impact human health.

2. MATERIALS AND METHODS

2.1 Zebrafish Culture

2.1.1 Zebrafish Adult Colony

The zebrafish adult colony consisted of approximately 1000 adult Tübingen wild type *D. rerio* obtained from the U.S. Army Center for Environmental Health Research (Fort Detrick, MD). Adult (>6 months of age) *D. rerio* were kept in 9 L housing units at a density of approximately 5 fish/L in equal sex ratios. Each rack system was maintained independently, and all breeding fish used in this study came from the same rack system.

2.1.2 Housing System

Each rack system was supplied with flow-through water generated from a well water source. Water was exchanged at a rate of approximately three turnovers per day. Water was circulated throughout the system via a 900 gal/h Danner pump (Danner Manufacturing; Islandia, NY). The colony was maintained at 28 °C in a semi flow-through Aquaneering (San Diego, CA) rack system with ultraviolet and fluidized silica bed biological filtration. Water parameters were consistently maintained via the flow-through system and recorded daily. Conductivity was maintained at approximately 300 μS/L; pH was maintained at 7.4–8.0, and dissolved oxygen was maintained at ~75–90%.

2.1.3 Diet

Our adult breeding fish were maintained on a high-quality laboratory diet of Gemma micro 300 feed (Skretting USA; Tooele, UT) which was provided daily via a metered Danio Lab (Boston, MA) feeding device. Twenty-four hours prior to egg production, their diet was supplemented with live, freshly hatched *Artemia* nauplii.

2.1.4 Egg Production

Egg production was synchronized to provide staged embryos for all experimentation. This was accomplished using the ISpawn-S system (Techniplast; West Chester, PA). The fish were separated by sex one day before the spawning event, and 15–18 females were placed in the breeding chamber and partitioned at the bottom. An equal ratio of males were placed above the partitioned females. Water at 28 °C was circulated through the system via a sump reservoir that contained carbon/floss filtration, a 300 W heater, and a 500 gal/h circulation pump. The total volume of the breeding system was approximately 80 L. It supported approximately 90 adult fish. Typically, 60 adult fish (30 males and 30 females) were split between two ISpawn-S systems with 30 fish per system. On the day of the spawning event, the partitions were removed, and water volume was lowered to simulate shoaling behavior at the time of the daylight cycle. Lights were gradually illuminated via an automated dimming system to simulate a natural sunrise over a period of 30 min. Spawning was halted by raising the water volume 1 h after the start of the spawn cycle. Eggs from fertile females were collected at the bottom of the breeding device and physically separated by the device from the spawning adults. Each spawning group was bred approximately every 8–10 days. Group performance records were maintained to ensure high fecundity. After the eggs were produced, they were collected via strainer and placed in a Petri dish with fish media. The volume of eggs produced was measured in milliliters and calibrated. The results showed that an average of 640 eggs/mL were produced for this strain. Fertile eggs were then placed in a 250–500 mL crèche. Air was bubbled through the crèche to keep the developing embryos circulating through the media until they hatched (48–60 h post-fertilization [HPF]).

2.1.5 Larval Culture

After they hatched, 60 HPF larvae were removed and placed in static tanks until testing commenced at 144 HPF. During that time, all larvae were maintained at a 14 h light cycle and 28 °C. Larval media were completely exchanged daily with fresh media (100%).

2.1.6 Larval Selection

For final larvae selection, we used a swim-over test to ensure all of the embryos had reached the same stage in development at 6 DPF. In brief, fish embryos were placed in a 90 mm Petri dish, which was then placed in a larger 140 mm Petri dish with a higher lip than the smaller dish. Both dishes were filled with water such that the level in the larger dish was raised above the lip of the inner dish. All larvae were placed in the inner Petri dish and allowed to swim freely for a minimum of 20 min. Only embryos that managed to swim over the lip of the inner dish to the larger one were selected.

2.2 Chemical Preparation/Dosing

All chemicals for toxicological assessment were prepared as either a 10 or 1 mM stock solution dissolved in dimethyl sulfoxide. Prior to testing, a 10 mM stock solution for either PT, PT-O, or VX was thawed at room temperature. Immediately before exposure, the 2× dosing solutions were prepared in 10 mL of embryo media, vortexed, and readied for dosing. After the appropriate dosing solution was prepared, larvae were dosed with 1 mL of a 2× solution of compound to make a final 1× solution of 2 mL.

2.3 Evaluation of Toxicity

2.3.1 Chemical Exposure of 6 DPF Larvae for 50% Lethal Concentration (LC_{50}) Determination.

The 6 DPF zebrafish larvae were plated in a 24-well Costar (Corning, Corning NY) nontreated polystyrene plate at a density of 10 fish per well in a 1 mL volume of fish media. Larvae were 1/2 log dosed .001–100 µM with a 2× solution of either PT, PT-O, or VX.

2.3.2 24 h Lethality (LC_{50}) Determination

The 24 h zebrafish LC_{50} determination was calculated from 30 larval replicates for each dose point. Concentration–response curves were fitted in GraphPad (Graphpad Software; San Diego, CA) using the non-linear regression (four parameter) equation: [Agonist] vs. response – Variable slope, $Y = Bottom + (XHillslope) \times (Top - Bottom)/(XHillSlope + ECHillslope50)$.

2.3.3 AChE Inhibition in 6 DPF Larvae After Chemical Exposure

Zebrafish larvae were plated in a 24-well Costar nontreated polystyrene plate at a density of 10 fish per well in a volume of 1 mL of fish media. Either 120 HPF (24 h time point) or 144 HPF (1 and 4 h time points) larvae were exposed to either 1.0 LC_{50} for each PT, PT-O, VX or 1 mL of fish media and incubated at 28 °C for either 1, 4, or 24 h. Larvae were removed from exposure media, placed in a 1.5 mL Eppendorf (Hamburg, Germany) tube, and frozen at –80 °C.

2.3.4 Modified Ellman's Assay for AChE Inhibition Analysis

In short, cleared homogenates were diluted 1:10 in deionized H_2O and buffered to pH 7.4 using 0.69 mM phosphate buffer. A 96-well Costar black assay plate was loaded with 200 µL of each sample in triplicate. To each well, 3 mM of 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent; MilliporeSigma; St. Louis, MO) was added to a final concentration of 300 µM and incubated for 10 min at 28 °C with shaking at 200 RPM on an orbital shaker. Just before analysis, 12.5 mM acetylthiocholine iodide and 1 mM 10-(alpha-dimethylaminopropionyl)phenothiazine was added to each well to a final concentration of 1 mM and 20 µM, respectively. The plates were then read on a Spectramax plate reader (Molecular Devices; San Jose, CA) using a kinetic assay to measure the change in

formation of 5-thio-2-nitrobenzoic acid anion at an absorption of 410 nm caused by the AChE hydrolysis reaction of Ellman's reagent.

2.3.5 AChE Inhibition Analysis (6 DPF Larvae)

Frozen samples for each time and chemical exposure condition were removed from the freezer (–80 °C temperature), thawed on ice, and manually homogenized in a mixture of tris-buffered saline and polysorbate 20 (TBST) pH 7.4 using disposable pestles. Ellman's assays were performed on each sample to determine the AChE inhibition via kinetic analysis using a Spectramax 384 plus spectrophotometer with Softmax Pro 5.2 (Molecular Devices). Eleven readings were captured with 20 s intervals between readings.

2.3.6 AChE Inhibition Analysis (6 DPF Larval homogenates)

Ten 6 DPF zebrafish larvae were placed in a 1.5 mL Eppendorf tube on a 4 °C ice bath for 20 min. All fish media were removed, and 100 µL of TBST pH 7.4 was added to each sample to be homogenized. Pooled larval homogenates were dosed to the equivalent of 1.0 LC_{50} of either PT, PT-O, or VX. Control samples were exposed to an equivalent volume of fish media. Each homogenate was incubated at 28 °C for either 1, 4, or 24 h. At the end of the timed chemical exposure, samples were frozen at –80 °C. Modified Ellman's assays were performed on each homogenate sample for the determination of AChE inhibition.

3. RESULTS

3.1 24 h Lethality of AChE Inhibitors in 6 DPF Zebrafish

VX was 1097× more lethal than PT and 134× more lethal than PT-O on the 6 DPF larval zebrafish (Figure 1). High variability was noted within the PT-O data. PT-O was unstable in an aqueous environment with a pH greater than 7.0.

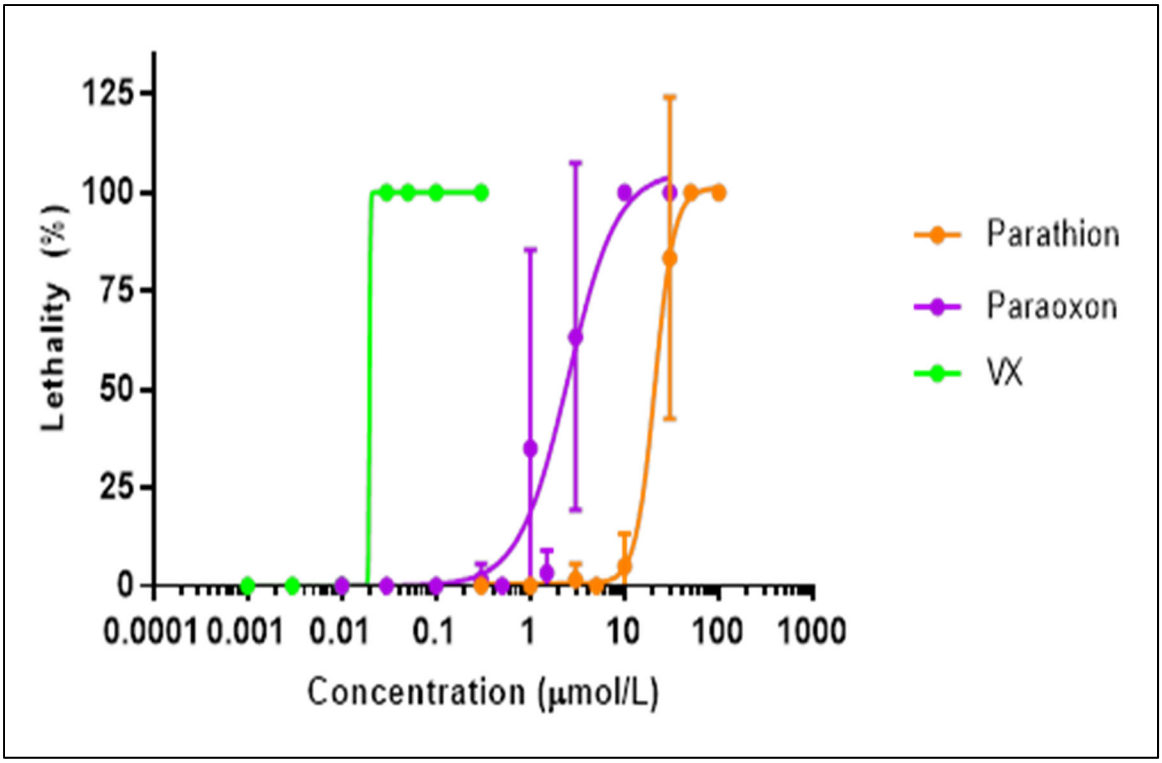

Figure 1. 24 h lethality of AChE inhibitors in 6 DPF zebrafish.
VX (observed LC_{50} of 0.019 µM) was 134× more potent than PT-O (observed LC_{50} of 2.54 µM) and 1097× more potent than PT (observed LC_{50} of 20.84 µM).

3.2 Time Course Evaluation of AChE Inhibition of 6 DPF Larvae Post 1.0 LC_{50} Exposure Compared with AChE Inhibition of 6 DPF Larval Homogenates Post 1.0 LC_{50} Exposure

In this evaluation, we compare the inhibition of AChE in exposed 6 DPF fish and exposed 6 DPF homogenates. Exposing 6 DPF larval homogenates provided a direct route to the liberated AChE in the homogenate. In addition, the typical pathways used to metabolize these compounds were no longer active within the homogenate. Figure 2A demonstrates the ability of the 6 DPF larval fish to metabolize the parent compound, PT, to its more potent AChE-inhibiting metabolite, PT-O. Figure 2B demonstrates that without the larval fish CYP metabolism, PT-exposed (average inhibition at 24 h = 0.44%) homogenates demonstrate minimal AChE inhibition after 24 h. Moreover, similar to VX, PT-O demonstrates the ability to directly inhibit AChE.

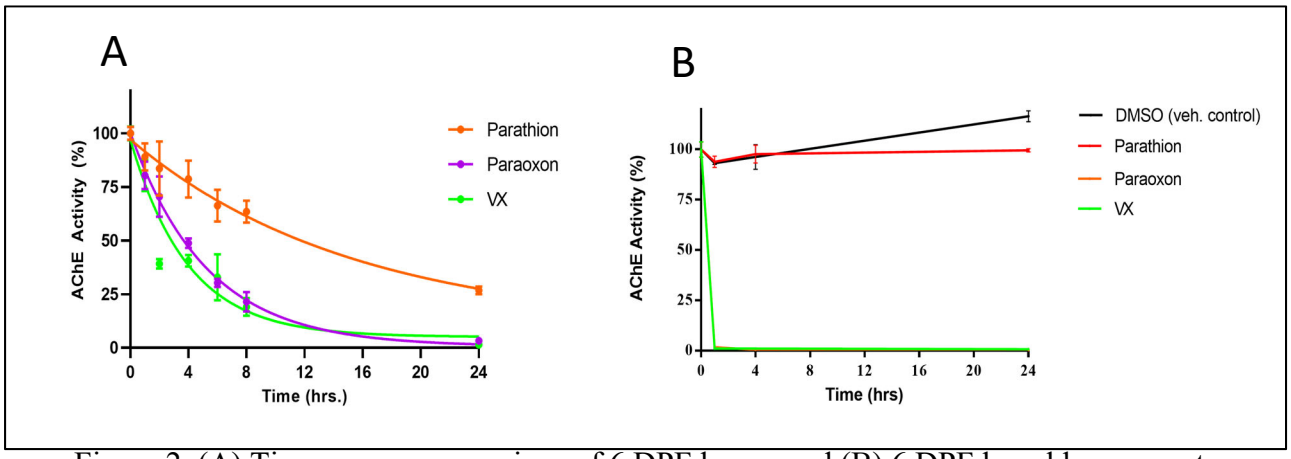

Figure 2. (A) Time course comparison of 6 DPF larvae and (B) 6 DPF larval homogenates exposed to 1.0 LC_{50} concentration of AChE inhibitors.

4. CONCLUSIONS

The persistence of chemicals in the environment has provided a multitude of avenues for human exposure. Tools that can quickly and accurately assess chemical hazards and risks are needed, especially when considering CWAs. OPs were of particular concern in this work due to their widespread use and chemical threat potential. Furthermore, OPs are of great human health concern because they disrupt key neurological functions that help maintain body homeostasis. Thus, high-throughput screening systems that can accurately assess these chemicals must be developed and validated. In this study, we aimed to develop an acute toxicity larval screening tool for AChE-inhibiting compounds using 6 DPF *D. rerio* embryos by examining the nerve agent lethality and AChE inhibition of PT, PT-O, and VX.

LC_{50} values were determined for VX, PT-O, and PT and reported as 0.019, 2.54, and 20.84 µM, respectively. Previous research has not assessed the LC_{50} values of these compounds using a 24 h exposure duration, although some studies have determined LC_{50} values and tested AChE inhibition for zebrafish using various other exposure timeframes.[5,20] In a similar study, 6 DPF zebrafish embryos were exposed to PT-O for <3 h, and an LC_{50} value 65× less potent (165.8 µM) than the LC_{50} reported here was observed.[5] LC_{50} values for PT have also been assessed in previous research. An LC_{50} value of 13.5 µM was reported for 16 h exposed 8 DPF embryos.[20] Although those results differ from the results reported here, the differences can be attributed to exposure methods, exposure times, and in the latter study, the age of the larvae. All of these studies demonstrate that, in the use of zebrafish for toxicological assessment, our model offers a simple approach for rapidly screening cholinesterase-inhibiting compounds to determine the relative potency of OP chemicals.

VX has been observed to be more toxic than PT-O in rats.[21] Our research supports this finding and allows for the use of a lower order model organism, such as zebrafish, as a high-throughput screening tool. This could impact the animal research community by reducing the need for higher-order animals. Moreover, previous VX testing on rats shows comparable LD_{50} values.[21,22] Zebrafish models maintain genetic proximity to humans but allow for faster, lower cost studies than rodent models.[23] Zebrafish are a well-known model organism in biochemical

research with a variety of studies using them for models assessing neurotoxicity, cardiotoxicity, and specific to this research, OP exposure.[23] The ease of models using zebrafish compounded with their homologous expression of AChE,[24] make zebrafish an excellent model for OP-related research.

In conclusion, we have demonstrated the validity of using the 6 DPF zebrafish larval model to evaluate OP toxicity. This model demonstrates the ability to determine the relative potency of the AChE-inhibiting compounds as seen with OP pesticides and their metabolites compared with the more potent CWA, VX. At lethal concentrations, the OP pesticide, PT, requires metabolism of the -oxon metabolite to inhibit AChE in exposed larvae. This indicates that the lethal effects of these compounds are not due to the activity of the parent compound on the cholinergic system and that the 6 DPF zebrafish larvae are capable of the biotransformation required to activate these types of compounds through hepatic CYP metabolism. Therefore, this model provides a more useful screening tool for potential nerve agent AChE-inhibiting compounds and can detect compounds that require metabolism to a toxic metabolite in a much more efficient high-throughput platform.

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ACRONYMS AND ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase enzyme
APG	Aberdeen Proving Ground
CP	chlorpyrifos
CWA	chemical warfare agent
CYP	cytochrome P450
DPF	days post-fertilization
HPF	hours post-fertilization
HTS	high-throughput systems
LC_{50}	50% lethal concentration
MT	malathion
OP	organophosphorus
PT	parathion
PT-O	paraoxon
TBST	Tris buffered saline (.1% Tween 20)
VX	*o*-ethyl s-diisopropylaminomethyl methylphosphonothiolate

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