Integrated Approach for Identifying the Molecular, Cellular, and Host Responses to Chemical Insults

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We performed a pilot study to characterize the molecular, cellular, and whole-organism response to nonlethal chemical agent exposure in the central nervous system. Multiple methodologies were applied to measure in vitro *enzyme inhibition, neuronal cell pathway signaling, and* in vivo *zebrafish neural development in response to challenge with two different classes of chemical compounds. While all compounds tested exhibited expected enzyme inhibitory activity against acetylcholinesterase (AChE), a well-characterized target of chemical agents, distinct differences between chemical exposures were detected in cellular toxicity and pathway target responses and were tested in a zebrafish model. Some of these differences have not been detected using conventional chemical toxicity screening methods. Taken together, the data demonstrate the potential value of an integrated, multimethodological approach for improved target and pathway identification for subsequent diagnostic and therapeutic biomarker development.*

INTRODUCTION

To build capability and leverage new and growing biology and chemistry expertise at APL, a collaborative, cross-departmental effort was established through a series of related independent research and development (IR&D) projects. The focus of this effort was on mitigation of chemical and biological threat agents. Biological model systems have varying degrees of complexity, from single-component biochemical experiments to whole-cell models to complete living organisms. Regardless of the model selected, challenges exist in sample collection, dose determination, and biases inherent in each assay/technology. Therefore, multiple experimental methodologies brought to bear on a particular biological question may facilitate more nuanced and insightful evaluation than any single method could in isolation. Guided by current research thrusts within the DoD, particularly

the Defense Threat Reduction Agency (DTRA), we initiated a pilot study aimed at characterization of the biochemical and biological consequences of chemical insults. Many of the chemical threats of interest within the community primarily target the nervous system, specifically acetylcholinesterase (AChE), an enzyme that mediates many neurological and neuromuscular interactions.[1,2] Several common pesticides similarly inhibit AChE.[3] In this study, we selected several of these pesticides to serve as model chemical agents and evaluated them against potential single-enzyme targets. Additionally, we chose an *in vitro* neuronal cell model to identify secondary cellular targets and pathways affected by these pesticides. Finally, we investigated AChE inhibitors in the context of the zebrafish as a whole-organism model system.

To complete the effort outlined above, a number of new tools needed to be implemented at APL. We envisioned this effort as an iterative biological characterization engine, as depicted in Fig. 1a. Data collection can start at any point along this path. Our studies began with single-enzyme inhibition screening of AChE inhibitors. Data from these experiments yielded detailed information about the relationship between molecular structure and biochemical interaction with single specific enzymes (protein catalysts).

The next level of increased biological complexity is the cellular model. Our work was performed in a human neural cell model, which was selected because pesticide compounds are known neural system effectors.[4] General pathways for a small molecule interacting with a cell are shown in Fig. 1b. A small molecule can interact with biochemical targets either inside or outside the cell and can affect pathways that lead to changes in gene expression or pathways that result from changes in gene expression. All of these effects have a temporal component, and multiple interactions can occur over the experimental time frame. These interactions can be characterized by several different techniques. Cell signaling (the process by which cells turn gene expression on or off) can be characterized by phospho-flow cytometry, a method for counting individual cells and assessing the state of the cell in response to the challenge. Multiplexed flow

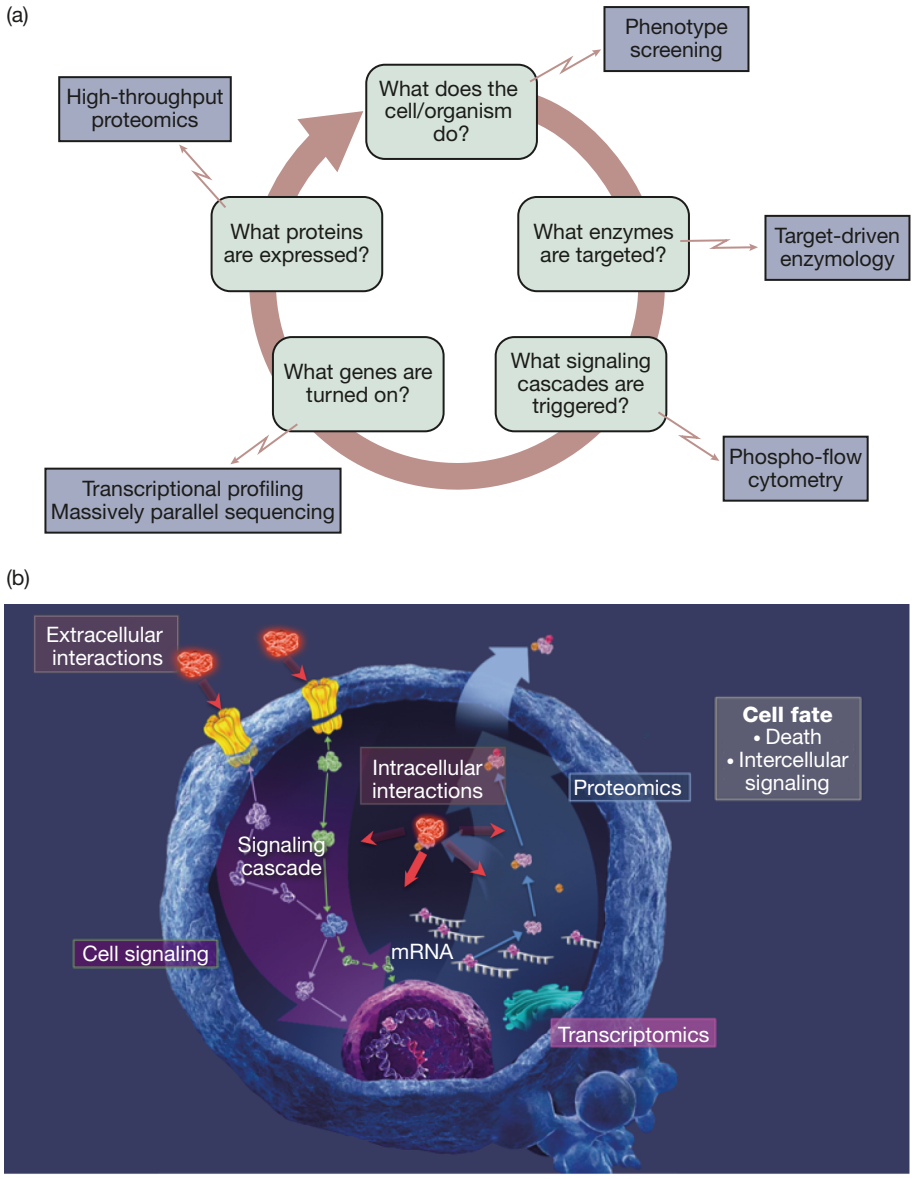


Figure 1. (a) Overview of a systems biology chemical insult characterization engine. Central questions asked during the characterization of a chemical insult appear in the circular engine in green boxes. Methodologies used for addressing each engine component question are linked by arrows. (b) Schematic representation of cellular responses to a chemical challenge. Intracellular interactions following extracellular challenge are represented and indicated with corresponding methodologies used to measure the response. Arrows indicate the temporal flow of events in the cell following challenge.

cytometry enables quantification of multiple cellular responses by using a broad dynamic range of fluorescence to detect multiple analytes simultaneously.

Mass spectrometry is the workhorse technology for proteomics, and we conducted a preliminary proteomic evaluation of carbamate AChE inhibitors in the neuronal model. Molecular interactions characterized by proteomics give rise to changes in cell state, which can be observed in a variety of phenotype assays. A phenotype can be suggestive of a molecular cell response but does not indicate the exact biochemical interactions that are occurring. Assays that evaluate phenotype are nonetheless extremely useful because they provide a link between molecular events in the cell and the gross cellular response. In our pilot study, neuronal cell phenotypes evaluated included cell viability and calcium flux. Viability provides an overall measure of cellular health under conditions of chemical treatment, and calcium flux is indicative of electrochemical response to chemical insult.

The neuronal cell model was useful for pathway analysis and cataloging molecular and cellular responses to chemicals, but it does not enable organ system–level investigation. Particularly for nervous system effectors, intact neural systems are critical to understanding biological responses. At the whole-organism level, we conducted a study of the effects of the carbamate class of AChE inhibitors in zebrafish. The zebrafish is an accepted vertebrate developmental model, and it is extremely useful for the study of the nervous system.[5] Zebrafish embryos are transparent for the first 5 days after fertilization, making imaging studies of body systems facile. In our experiments, both gross phenotypic changes in zebrafish embryos and compound-specific effects on the developing nervous system were characterized.

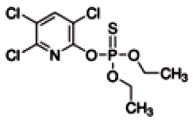
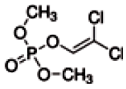
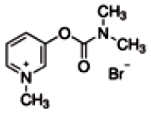
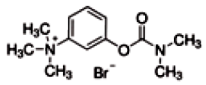
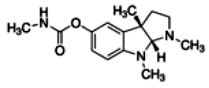
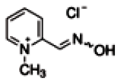
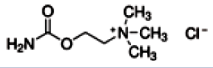
EXPERIMENTAL APPROACHES AND RESULTS

Experimental results from each of the efforts outlined above are provided as an overview of major experimental findings without extensive experimental detail.

Single-Enzyme Studies of Small-Molecule Enzyme Inhibitors

Although AChE is the primary target of toxic phosphate esters, secondary targets including enzymes, neurotransmitter receptors, and cellular structural proteins may also have a significant role in nerve agent pathology.[6–8] Many enzymes other than AChE are also hypothesized organophosphate (OP) binding proteins, including fatty acid amide hydrolase (FAAH), mono-

Table 1. Panel of enzyme assays used to characterize both AChE inhibition and inhibition of potential secondary targets of AChE inhibitors

Compound	Use	Class	Structure	AChE IC_{50}	MAGL IC_{50}	FAAH IC_{50}
Chlorpyrifos	Pesticide	OP		524 μM	N.D.	N.D.
Dichlorvos	Pesticide	OP		300 μM	4712 μM	3.4 μM
Pyridostigmine bromide (PB)	Prophylactic	Carbamate		6.6 ± 1.7 μM	>10,000 μM	>10,000 μM
Neostigmine bromide (NB)	Prophylactic	Carbamate		330 ± 30 nM	>10,000 μM	>10,000 μM
Physostigmine (PS)	Prophylactic	Carbamate		870 ± 20 nM	>10,000 μM	>10,000 μM
2-Pralidoxime	AChE reactivator	Oxime		—	—	—
Carbachol	AChR activator	—		—	—	—

AChE was purified from electric eel used for *in vitro* enzymatic inhibition experiments. IC_{50} denotes the concentration at which 50% of the target has been inhibited. N.D., not detectable.

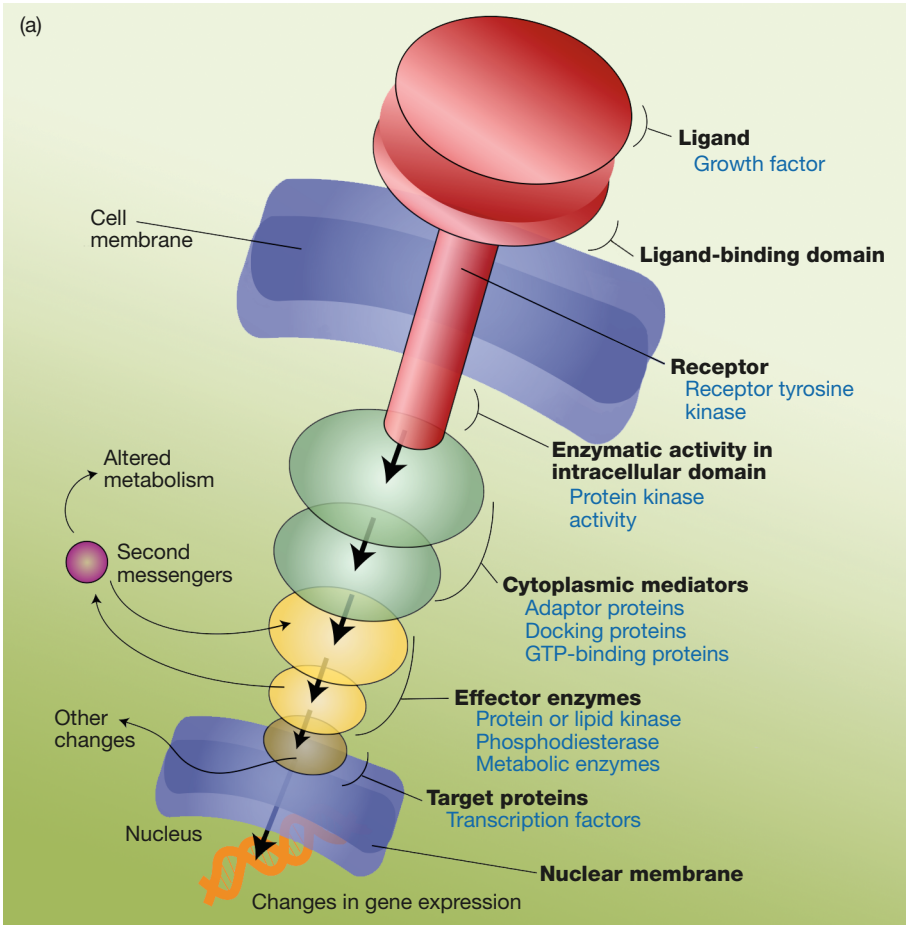


Figure 2. Differential effect in phospho-epitope profiling between different AChE inhibitors. (a) Schematic of a generic signal transduction pathway. Bold text shows components of signaling pathways; blue text lists specific examples. Receptors on the extracellular surface bind to ligands stimulating conformational changes throughout the pathway. (b) SH-SY5Y cells were treated with 100 μM chemical and fixed 5 min post exposure. Cells were stained with antibodies against various phospho-proteins. Heat map visualization was generated to analyze mean fluorescence intensity values and capture fold changes above or below basal levels of phosphorylation of target proteins listed on the left panel of the heat map.

acylglycerol lipase (MAGL), neuropathy target esterase, paraoxonase, KIAA1363, and others.[8] We developed a panel of enzyme assays to characterize both AChE inhibition and inhibition of potential secondary targets of AChE inhibitors (data are shown in Table 1). MAGL and FAAH mediate endocannabinoid biosynthesis, and inhibition of both of these enzymes leads to neurological impairment. The need to evaluate these two enzymes arose during the DTRA-funded Simulant Down Select Program, under which OP nerve agent simulants were developed. These small molecules had no AChE inhibitory activity, but there were concerns about potential off-target effects in the endocannabinoid system.

For completeness, Table 1 also includes carbamate drugs as well as additional cholinergic system modulators. Pyridostigmine bromide (PB), neostigmine bromide (NB), and physostigmine (PS) are carbamate drugs that can be used as prophylactic AChE inhibitors.[2] Pralidoxime is an oxime AChE reactivator: it reacts with carbamate-bound AChE to release a reservoir of AChE in the event of nerve agent exposure. Carbachol is a nicotinic acetylcholine receptor (nAChR) modulator, which is discussed in greater detail below.

Profiling Cellular Protein Signaling

Signaling proteins operate within a cell through changes in post-translational modifications and covalent addition or removal of a variety of functional groups, including phosphates, carbohydrates, sulfates, and others. Measuring the

phosphorylation status of specific proteins can identify which signaling pathways have been activated (schematically represented in Fig. 2a). Protein phosphorylation can be detected using flow cytometry, a technique by which cells are tagged with fluorescently labeled antibodies that specifically bind phosphorylated sites on proteins.

In this study, we assessed the differential effect in protein-phosphorylation events following OP and carbamate challenge in our neuronal cell model, SH-SY5Y. These cells were derived from a human neuroblastoma cell line and are widely used as a neuronal model. Modification of proteins within known acetylcholine receptor (AChR)–related pathways was measured in a very short time frame (5 min), as signaling changes are thought to occur immediately following exposure, leading to intermediate and longer-term effects as measured by phenotypic assays described below. Differences in protein-phosphorylation in neuronal cells were observed following chemical treatment. Figure 2b is a heat map representation of multiple experiments showing relative fold changes in phosphorylation of signaling proteins thought to be involved after chemical challenge. While these experiments suggest that structurally similar OPs can exhibit differential signaling signatures, further experimentation needs to be performed to draw definitive conclusions on the exact pathways affected and the intended secondary (non-AChE) targets.

Phenotype Assays

The term phenotype refers to the observable characteristics of a biological entity such as morphology, growth, behavior, death, and other physical traits. Overall, measuring cell viability is a good "first pass" experiment for evaluating the phenotypic effects of a challenge with any small molecule. The effect of selected OPs and carbamates was measured in our human neuronal cell line using a colorimetric cellular viability assay, which measures metabolic activity after chemical exposure. Lack of metabolic activity in a cell population is indicative of cell death. Because both chronic and acute OP exposures have been shown to induce cellular death in relevant literature, we determined that it was necessary to examine effects over a broad dose range (0–1000 μM).[9–11] Figure 3 shows the titration curve of the OP and carbamate effects on cellular viability and is expressed as a percent of control samples. While PB, NB, and chlorpyrifos (CPF) were not shown to have any significant effects toward decreasing viability over the concentration range tested, PS and dichlorvos (DDVP) exposure did result in a significant decrease in viability. These effects occurred at concentrations significantly higher than the concentration necessary to inhibit AChE, indicating that a secondary mechanism is probably involved. In fact, delayed neurotoxic effects have been documented in individuals after acute exposure to OP, a condition termed OP-induced delayed neuropathy.[12] Similarly, this condition is not thought to occur because of effects on AChE itself.[4] To avoid this specific acute response in our model, all subsequent experiments were performed at subtoxic doses.

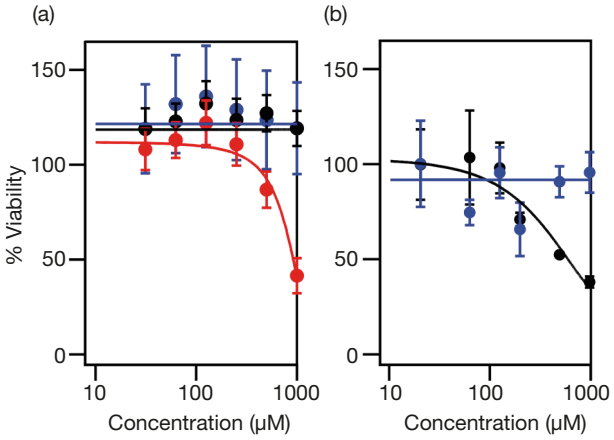


Figure 3. Cellular toxicity following overnight incubation with AChE inhibitors. SH-SY5Y neurons were incubated with varying concentrations of (a) OPs and (b) carbamates for 18 h before evaluation of toxicity. (a) Viability following carbamate incubation was investigated for PB (black circles), NB (blue circles), and PS (red circles); and (b) viability following OP incubation was investigated for DDVP (black circles) and CPF (blue circles). Percent viability was calculated from the signal ratio between the control and treated samples; percent viability measurements greater than 100% indicate continued cellular growth. The toxicity IC_{50} was 500 μM for DDVP and 900 μM for PS; data were fit to sigmoidal curves (number of experiments $n = 3$).

Ion flux within cells is also an important indicator of cell response mechanisms to chemical exposures. Calcium (Ca^{2+}) is a critical signaling messenger within cells; it regulates processes as diverse as fertilization, proliferation, development, learning, memory, contraction, and secretion. In our experiments, neuronal cells were challenged with chemicals over a wide range of concentrations and subsequently treated with carbachol, which stimulates the AChR on the cell surface. This stimulation can occur via two distinct types of cell surface receptors, muscarinic and nicotinic, both expressed by our cellular model.[13–15] Since stimulation of AChE receptors causes a Ca^{2+} ion flux via two mechanisms, the broad effects of chemical agents within the cell can be estimated. Fluorescent Ca^{2+} indicators (termed Fluo-4) were used to measure changes in signaling in the neuronal model cell line. All three compounds tested altered the Ca^{2+} signal in a dose-dependent manner (Fig. 4). Cell challenge with CPF and PB induced an increased release of Ca^{2+} ions in the cell, whereas the DDVP inhibited Ca^{2+} release as compared with untreated controls. These data indicate that while both classes of compounds interact with components of the intracellular signaling pathway, they do not all interact with the same binding partners.

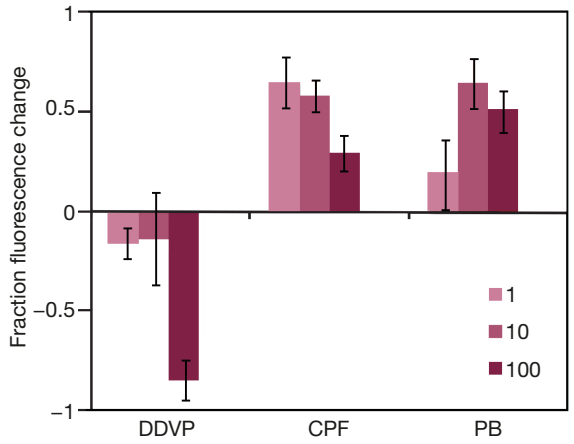


Figure 4. Changes in Ca^{2+} flux in response to OP and carbamate exposure. SH-SY5Y neurons were incubated with varying μM concentrations of OPs (DDVP and CPF) and carbamate (PB) for 18 h before evaluation of Ca^{2+} release in response to carbachol stimulation. Data represent control normalized fluorescent signal change over 500 s read time frame ($n = 3$).

Proteomic Profiling of PB-Exposed Neuronal Cells

Mass spectrometry–based proteomics offers a powerful method for quantification of protein populations within cells, enabling researchers to capture a broad protein "snapshot" of a particular condition of the cell. In collaboration with Professor Akhilesh Pandey at the Johns Hopkins School of Medicine, we conducted proteomic profiling of carbamate-treated neuronal cells as a pilot study. On the basis of cellular and enzyme inhibition data, we characterized untreated neuronal cells and neuronal cells treated with 10 μM PB. Proteins that were up-regulated (more expression) and down-regulated (less expression) in PB-treated cells are shown in Fig. 5. Whereas only 16 proteins were up-regulated by 10–50%, 85 proteins were down-regulated by 150–200%. The up-regulated proteins were primarily gene expression functioning, i.e., nucleic acid binding and transcription factors. In contrast, the down-regulated proteins comprised mostly cytoskeletal, signal transduction, transporters, and metabolic process proteins in addition to a significant portion of gene expression regulators. Because this was a preliminary study, the temporal changes in protein expression levels are not captured by this data set. Characterizing the trajectory of protein changes over multiple time points will provide more complete information about cellular responses to chemical challenge.

Zebrafish Toxicology Studies

As model organisms, zebrafish have a number of advantages relative to mammalian models. Zebrafish embryos develop rapidly outside of the female in a transparent egg, allowing manipulation and visualization throughout development. Because the embryos are transparent during development up to 5 days post fertilization (dpf), all organs can be clearly seen and investigated without euthanizing the animal. The embryos can be generated in large quantities for high-throughput experiments, and a large number of transgenic lines are available for mutational studies.

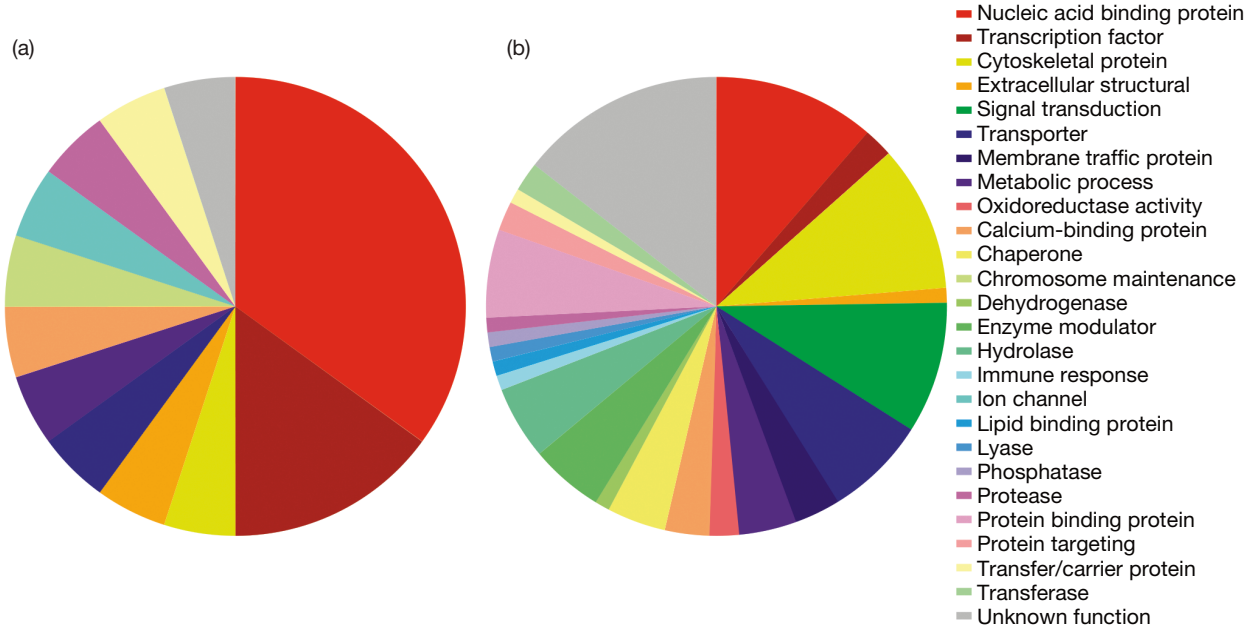


Figure 5. PB exposure induces up-regulation as well as down-regulation of proteins within neurons. SH-SY5Y cells were treated with 10 μM PB and lysed 24 h post exposure. Results were categorized by protein class and visualized by pie chart for (a) up-regulated proteins and (b) down-regulated proteins. Panther classification system (http://www.pantherdb.org) and NCBI Gene were used to classify proteins.

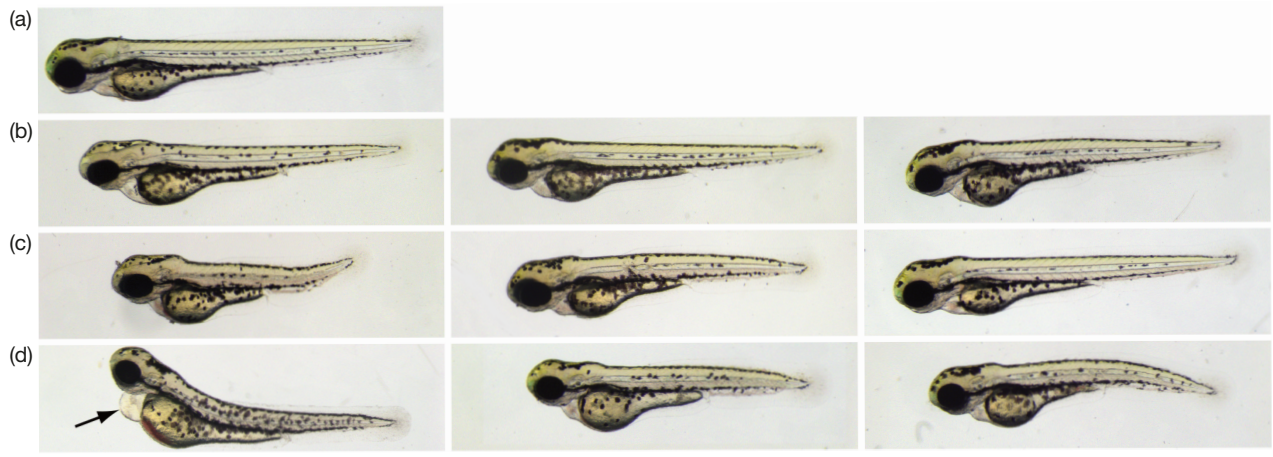


Figure 6. Gross morphological phenotypes resulting from chronic carbamate dosing. Representative images of 3 dpf embryos: (a) salt-water control; (b) embryos treated with PB at 100 mM (left), 50 mM (center), and 10 mM (right); (c) embryos treated with NB at 100 mM (left), 50 mM (center), and 10 mM (right); and (d) embryos treated with PS at 0.5 mM (left), 0.1 mM (center), and 50 μM (right).

Zebrafish have been previously used as a tool for evaluation of small molecules that affect the nervous system. CPF has been previously demonstrated to elicit neurobehavioral effects and corresponding impairment of sensory neuron development in zebrafish.[16,17] Treatment of zebrafish with edrophonium or tacrine, both FDA-approved drugs and reversible AChE inhibitors, causes sensory neuron formation in incorrect locations within the fish.[18] In the current study, we evaluated the toxicity and developmental effects of three previously uncharacterized carbamate AChE inhibitors—PB, NB, and PS—on zebrafish.

Phenotype of Carbamate-Exposed Zebrafish

The physical or phenotypic effects of AChE inhibition on development of zebrafish embryos were investigated. Embryos were dosed daily starting 3 h post fertilization (3 hpf), and the full range of affected organ systems was characterized. Embryos were treated daily for 5 days with a range of concentrations of PB, NB, and PS to evaluate the dose dependence of the phenotypic effects (Fig. 6). Compound stability over the 24-h incubation period was confirmed by mass spectrometry (data not shown). We observed a range of phenotypes distinct from embryos incubated in seawater media alone (Fig. 6a).

PB exposure induced the mildest effects of the three compounds (Fig. 6b, left panel), and embryos exposed to the lower two concentrations of PB developed normally (Fig. 6b). NB exposure induced stunted and curved body line for all three conditions evaluated (Fig. 6c). The highest dose of NB also slowed the heartbeat, swelled the yolk sac, and induced continuous twitching (Fig. 6c). Similarly, PS exposure induced stunted and curved body line, and a variety of other effects, for all three doses (Fig. 6d). Swimming was also impaired by persistent PS exposure.

Acute Carbamate Exposure Induces Impaired Motility

Swimming requires a functional nervous system, and impairment can be characterized in a zebrafish acute exposure assay for inhibition of AChE.[18] Immediate effects of AChE inhibitors on the nervous system were observed after 1-h chemical exposures without generat-

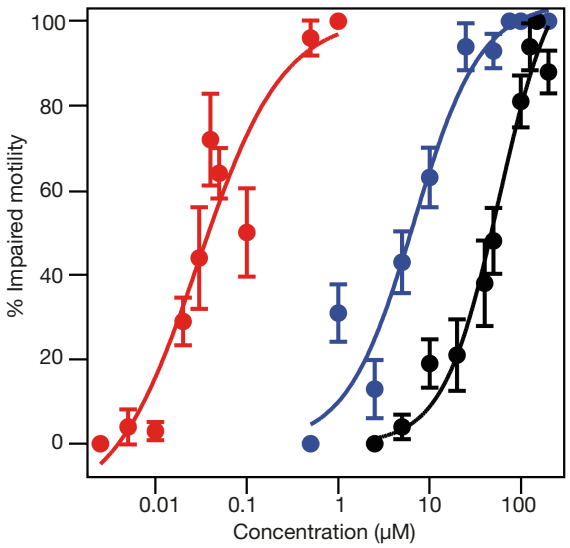


Figure 7. Motility impairment following acute drug exposure. Six-day-old larvae were incubated with PB (black circles), NB (blue circles), and PS (red circles) for 1 h before evaluation of motility impairment over a 60-s time period (performed in triplicate). The data presented represent the average percentage of larvae that exhibited any form of impaired motility, i.e., twitching, two-dimensional restricted swimming pattern, or reduced active swimming time. The IC_{50} values of zebrafish swimming inhibition was determined to be 60 ± 20 mM for PB, 10 ± 2 mM for NB, and 30 ± 10 μM for PS. IC_{50} values were calculated from the midpoint of Hill equation fits, and the average *n* per condition was 34.

ing the more systemic phenotypes. The fish exposed to the lethal compound dose (determined from phenotype studies) exhibited continuous twitching (Fig. 7). This effect was dose dependent, and, as in the *in vitro* AChE inhibition studies, PB was the least potent of the three compounds. The concentration at which 50% of the enzyme was inhibited (IC_{50}) was determined for each compound, and those concentrations were used for subsequent experiments.

Carbamates Have Differential Effects on Neuronal Development

We characterized the effects of PB, NB, and PS exposure on nervous system development by using transgenic zebrafish that express green fluorescent protein (GFP) in the motor neurons. A typical neuron possesses a cell body (soma), an axon (major branch extending outward from the soma up to 1 mm in length), and dendrites (thousands of smaller extensions outward from the axon about a micrometer in length). The impact of a sublethal dose of each compound was investigated for the four main steps during neuronogenesis: neuron cell body formation, axon formation, dendrite outgrowth, and synapse formation between motor neuron and muscle cells. Under all conditions tested, motor neuron cell bodies developed similarly to the control, as illustrated in the pronounced formation of many cell bodies clustered in a horizontal line near the top of each image (Fig. 8a). Incubation with PS resulted in degradation of the axon after 3 dpf (Fig. 8a). Axons grow along the edge of each segment of the fish; each chevron is formed from axonal extensions of the primary motor neurons (Fig. 8b). Careful evaluation of the angle of the chevron reveals the effects of PS and NB on musculature development. The NB- and PS-treated zebrafish show chevron angles that are wider than in control fish, making the segments (somites) of the fish shorter than those treated with the PB or vehicle control. This accounts for the significantly shortened body line monitored in the treated fish.

Once the axon has fully extended, small dendrites begin to branch out, a process called arborization. Treatment with PB did not induce significant disruption of arborization; however, exposure to NB and PB resulted in increased numbers of dendrites sprouting along the full length of the axon rather than along the bottom third of the axon (Fig. 8a). Finally, we examined the effects of carbamate exposure on synaptogenesis (synapses form at the interface between postsynaptic termini on muscle cells and dendrite extensions on presynaptic neurons). Staining of the control samples shows nAChR expression along the segment chevrons, neuron axon,

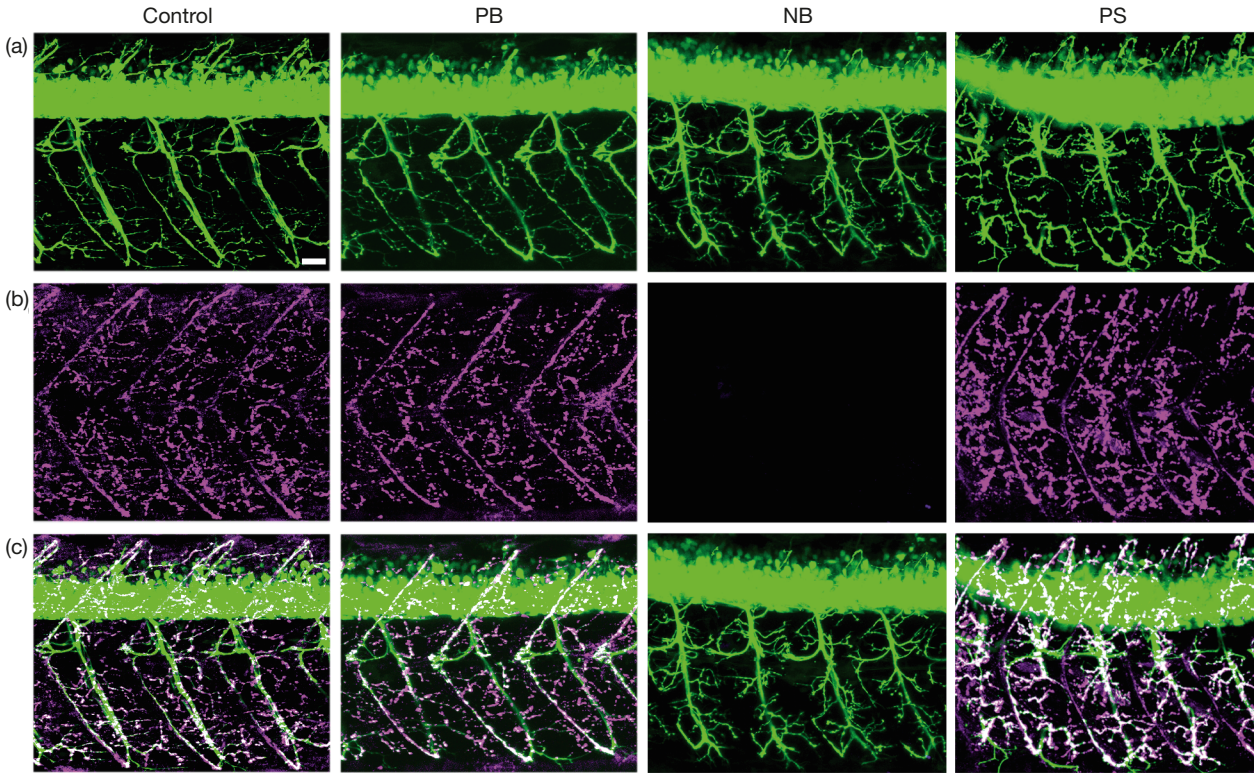


Figure 8. Chronic carbamate exposure effects on neurogenesis. Representative images of treatment with sublethal AChE inhibitory concentrations of PB, NB, and PS starting at 3 hpf with daily dosing. (a) Embyros (3 dpf) expressing GFP in motor neurons were stained with modified Cy5, which binds to (b) nAChRs, and (c) assayed for synapse formation at 3 dpf. Overlay of (a) and (b) to show colocalization/synapse formation is illustrated in white ($n \geq 30$, six images taken per condition).

and small punctuate structures throughout the muscle fibers, a pattern characteristic of native nAChR expression (Figs. 8b and 8c). PB and PS treatment resulted in control-like synapse formation. However, NB treatment consistently resulted in a mislocalization of nAChRs (Fig. 8c). Although the effective concentration of each compound was equivalent behaviorally and biochemically, each induced a distinct profile of phenotypic and neuro-inhibitory defects.

DISCUSSION OF EXPERIMENTAL RESULTS

This initial effort brought together a multidisciplinary team and resulted in interesting new observations regarding exposure to OP and carbamate chemicals. The integrated approach taken here highlights differential cellular viability and signaling effects following chemical exposure to AChE inhibitors. In our pilot study, the least complex systems were single enzymes; the most complex system was the zebrafish, a whole organism. With increasing complexity, the precision of the observations decreases, but system-wide effects can be observed. Thus, from single-enzyme experiments, subtle structure-based differences in protein–small molecule interactions can be gleaned. However, only upon moving to the zebrafish model could information about the effects of this series of small molecules in intact nervous systems be gathered.

Only two of the pesticides tested in our neuronal model resulted in a significant decrease in overall cell viability. These effects in the cellular system were measured at much higher dosages than what was expected from the direct biochemical inhibition of AChE. This observation points toward a secondary mechanism of cell death by acute exposure to neurotoxins, independent of AChE inhibition. Similarly, delayed neuropathy has been documented in acutely exposed individuals and is not thought to be due to direct AChE inhibition.[12]

Because stimulation of AChE receptors has been shown to cause cellular Ca^{2+} ion flux, we examined the broad effects of our model chemical agents on this signaling molecule (Fig. 4). Though all three compounds tested were shown to alter the Ca^{2+} signal in a dose-dependent manner, CPF and PB induced an increase of Ca^{2+} ions in the cell, while the DDVP inhibited Ca^{2+} release. These observations indicate potential mechanistic differences in the way these compounds interact with cellular targets. Similarly, differential protein phosphorylation was observed in the phospho-flow cytometry experiments following carbamate and OP exposure (Fig. 2). Evaluation of the PB-treated neurons by proteomic analysis revealed a significant down-regulation of signal transduction and gene expression proteins after PB exposure (Fig. 5). These preliminary data suggest that these chemicals, even the structurally similar OPs, interact differently along cell signaling pathways. Further elucidation of these agent-specific mechanisms could potentially be used to inform diagnostics by indicating unique signaling profiles.

During this study, we were able to extend our experimentation to whole-organism testing. Use of this zebrafish model allowed us to define the morphological phenotype of carbamate exposure (Figs. 6 and 7). These effects were dose dependent, and, as in the *in vitro* AChE inhibition studies, PB was the least deleterious of the three compounds. From these studies, we determined that each compound, though equivalent behaviorally and biochemically, induced a distinct profile of phenotypic and neuro-inhibitory defects (Fig. 8).

Translating inhibitory concentrations across assays is a critical aspect of integrating the individual data sets. The differential compound sensitivity monitored in the results presented here is, in large part, attributed to the large range of assay types. *In vitro* enzyme assays are classically considered the most sensitive, as the reaction mixture contains only the necessary components. In contrast, cellular assays are often less sensitive, as the compound may have difficulty crossing the cellular membrane and may be adsorbed by other nonspecific interactions. Animal studies suggest similar absorption efficiency differences, a characteristic that illustrates the effectiveness of a compound to serve as a drug. This is highlighted by the zebrafish motility experiments in which micromolar PS induces impaired motility, NB alters motility in the millimolar range, and *in vitro* studies with zebrafish (data not shown) and electric eel AChE (Table 1) show similar inhibition for NB and PS in the submicromolar range.

Overall, this study indicates that chemicals with the same primary molecular target (AChE) can have differential effects on cellular viability, signaling, and overall organismal health and function. The integrated approach chosen for this work resulted in the generation of several independent data sets, which in combination suggest multiple secondary effects of OPs and carbamates, beyond interaction with AChE. Traditional paradigms for hazard identification and risk assessment for chemical agents are based on toxicity tests using *in vivo* (typically rodent) models. While these models are designed to generate relevant data specific to adverse outcome (e.g., cancer, neurotoxicity, and reproductive toxicity), they are impractical for screening large numbers of chemicals because of resource cost and requirements. *In vivo* models also do not identify secondary and off-target mechanism(s) of action. New paradigms for testing are emerging that favor the use of *in vitro* cell-based models to provide higher-throughput screening such that the results of the screening would aid in the prioritization of resources and potentially better targeting of mechanism of action through extrapolation from the cell-based model.[19,20] However, whole-animal models still remain a critical experimental component, particularly for threat

agents that elicit effects in multiple systems that cannot be adequately modeled in cell culture.

TOWARD A SYSTEMS BIOLOGY APPROACH

Systems biology is an emerging field that holds great promise for improving our understanding of complex biological responses and interactions within a cellular system after exposure to an environmental challenge. In the past 10 years, the field of systems biology has emerged as a comprehensive and quantitative approach to investigate the manner in which all of the components of a biological system interact functionally over time.[21–23] At a practical level, a systems biology approach must capture and integrate global sets of biological data from as many hierarchical levels of information as possible. These could include DNA sequences, RNA and protein measurements, protein–protein and protein–DNA interactions, biomolecules, signaling, and gene regulatory networks, cells, organs, individuals, populations, and ecologies. The data are then transferred to comprehensive databases, where they are warehoused and annotated. New bioinformatics and computational tools have been developed to convey properties and behaviors of the system through the use of visualization maps and cell pathways. Systems biology should not be confused with systems engineering, which focuses on complex engineering project design, work process, and life cycle management. Systems biology seeks to simultaneously evaluate and integrate multiple levels of biology and biochemical interaction, providing insight from the molecular level to the whole-organism system level.

Our pilot study represents a first step toward a systems biology approach to studying biological effects of threat agents. As APL's biological capabilities expand and strengthen, we will be poised to support future needs for rapid and robust target and pathway identification as well as diagnostic and therapeutic biomarker development.

CONCLUSIONS

Systems biology approaches are beginning to shed light on the complexity of the cell signaling events that lead to common symptomatic observations such as pain, fatigue, or neuropathy. Our experience highlights the challenges in data analytics presented by collecting multiple data sets from different technology platforms and model systems (*in vitro* and *in vivo*). Future data analysis efforts will require iterative data mining and fusion, followed by predictive model generation and refinement through experimentation. This pilot study focused on the effects of chemical exposures on the host because of the expected interest of DoD sponsors in identifying early signatures and distinguishers of chemical agent exposure. However, the process outlined here is general, and future studies could easily extend to characterizing the host responses to pathogen, chemical, and other types of exposures.

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