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Collaborative Core Research Program for Chemical-Biological Warfare Defense

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14. ABSTRACT
Classical chemical nerve agents (CNAs) and numerous emerging chemical threats (ECTs) collectively referred to as chemical warfare agents (CWAs) share acetylcholinesterase (AChE) inactivation as a common target for their respective modes of action. To date, there is no known countermeasure with therapeutic efficacy for rescuing enzyme activity against a broad-spectrum of CWAs. Therefore, the major thrust of the work being performed by the Biological Modeling Group (BMG) at AFRL and its collaborators at the University of Cincinnati's Metabolic Diseases Institute (UC-MDI) and the Battelle Memorial Institute's Biomedical Research Center (BBRC) is focused on developing novel, broad spectrum countermeasures involving small molecule allosteric modulators for AChE activity. The research team hypothesizes that by utilizing allosteric modulation of the enzyme activity, treatment before or shortly after CWA exposure would protect the enzyme from inhibition.

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Research Activities Summary from October 1 2012 to September 30 2014

Collaborative Core Research Program for Chemical-Biological Warfare Defense

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Work Unit #: ODTWP004

Objective: Classical chemical nerve agents and numerous emerging chemical threats collectively referred to as chemical warfare agents (CWAs) share acetylcholinesterase (AChE) inactivation as a common target for their respective modes of action. To date, there is no known countermeasure with therapeutic efficacy for rescuing enzyme activity against a broad-spectrum of CWAs. Therefore, the major thrust of the work being performed by the Biological Modeling Group (BMG) at Air Force Research Laboratory and its collaborators at the University of Cincinnati's Metabolic Diseases Institute (UC-MDI) and the Battelle Memorial Institute's Biomedical Research Center (BBRC) is focused on developing novel, broad spectrum countermeasures involving small molecule allosteric modulators for AChE activity. The research team hypothesizes that by utilizing allosteric modulation of the enzyme activity, treatment before or shortly after CWA exposure would protect the enzyme from inhibition.

Technical Approach: The technical approaches, contained within six tasks that enable discovery of new countermeasures with therapeutic efficacy for rescuing AChE enzyme activity against a broad-spectrum of known CWAs, for the above objective are described below.

Task 1: Drug Discovery through High Throughput Screening (HTS) and Fragment-Based Drug Design (FBDD)

Current pharmaceutical approaches involving drug discovery programs commonly leverage two complementary techniques, wet chemistry high throughput screening (HTS) methodology and an *in silico* chemical and protein structural analysis and docking program generally known as fragment based drug design (FBDD). The main advantage of using these approaches is that the wet chemistry results are linked to advanced structural chemistry selection and protein tertiary structural solutions to change the risk profile for success of follow-on chemistry. In FBDD, the predictable chemistry risk is highest early on because fragments bind weakly to the target, and balancing compound structural evolution is challenging. Whereas HTS, using a structurally diverse chemical library, can certainly yield a larger number of potent hits early on, these well-designed assays are sensitive to changes induced by solution and chemical modifications; the molecular size is such that general binding to the target is insensitive to those same modifications. Both techniques are typically carried out in parallel to mitigate the inherent risks of not identifying strong chemical-protein target binders and not delivering satisfactory chemical starting points for lead optimization. Using FBDD, desired drug-like physicochemical properties can be incorporated at early stages, increasing the likelihood of success downstream in the pipeline. Hits identified from either screening technique were transitioned to Task 2 for confirmation of binding and optimization.

- *Task 1.1 – Candidate selection via HTS*: Traditional HTS using a modification to the colorimetric AChE activity method known as the Ellman's assay was performed to screen 100,000 drug-like molecules from the Proctor & Gamble proprietary library at the University of Cincinnati's Drug Discovery Research Center (UC-DDRC, part of UC-MDI). Using an EvoTec robot, single point (10 μM final solution test chemical concentration)

measurements were screened in HTS fashion to identify AChE activating compounds. Historically, the UC-DDRC has found that approximately 800 confirmed hits from their diverse chemical library will be identified from this HTS.

- *Task 1.2 – Candidate selection via FBDD*: In collaboration with the Ohio State University (OSU), we proposed to design and purchase our own fragment library that represents available drug-like chemical space. High concentration screening (100 μM) were used to identify weak binding fragment molecules as starting points for combinatorial chemical development.

Task 2: Hit-to-Lead Transition and Lead Optimization

Initial chemical structural hits from screening must first be culled for false positives. By utilizing non-activity based methods, the compounds and fragments directly interacting with the enzyme were selected and further investigated.

- *Task 2.1 - Orthogonal assays to confirm candidate hits and identify lead compounds*: Confirmation of binding and elimination of false positives were performed using one of three biophysical techniques: surface plasmon resonance (SPR) (Biacore T200), isothermal titration calorimetry (MicroCal Auto-iTC200), and/or microscale thermophoresis (NanoTemper NT.115 Red/Green). Each of these methods provides complementary information regarding binding including dissociation constants, kinetic binding rates, and thermodynamic properties. Subsequent to single-point hit identification and orthogonal binding confirmation, an additional dose-response enzyme activity assay was performed using ten concentration points. This large number of data points allowed for determining half maximal effective concentrations (EC_{50}).

- *Task 2.2 – Evaluating AChE protection against agent-based inhibition*: Confirmed leads were screened for efficacy in preventing or reducing AChE inhibition using the same modification to Ellman's assay as in screening. A single concentration of the lead at six-fold above the EC_{50} was incubated with the enzyme prior to a dose-response inhibition assay using twelve concentrations of CWAs and/or analogs. Initial proof-of-concept was performed by the BMG using paraoxon as a surrogate before transition to BBRC for live agent testing (VX, sarin, soman and other agents upon request).

- *Task 2.3 – Lead optimization using scaffold-hopping*: Lead expansion ("scaffold-hopping") sets were created from the positive leads using OpenEye's Rapid Overlay of Chemical Structures (ROCs) and Electrostatic Similarity (EON) software on the Air Force Super Computer, Spirit. The software queried virtual chemical libraries, such as ZINC and eMolecules, totaling more than 10 million compounds for structural and electrostatic similarity to the leads. Using the results from activity and binding assays, quantitative structure activity relationships (QSAR) were made and lead candidates for intelligent design and combinatorial synthesis were selected. Early *in silico* absorption, distribution, metabolism, excretion and toxicity (ADMET) profiling using QSAR models reduced attrition rates of compounds and reduced the need for expensive, labor-intensive assays by profiling the pharmacokinetic (PK) properties of leads, including

predicting p-glycoprotein (P-gp) specificity, oral bioavailability, passive absorption, blood-brain-barrier permeation, distribution, P450 inhibition, and maximum recommended daily doses. These predictions were sent to medicinal chemists at OSU who synthesized 30-50 derivatives of our top lead candidates based upon these computational design principles for further activity screening and binding evaluation. This iterative process of lead optimization through testing, computational analysis and predictions, and *in vitro* evaluation yielded improved leads with a high chance of success within *in vivo* animal experiments and resulted in more likely transition of therapeutic Investigational New Drug (IND) candidates.

Task 3: Protein Biochemistry for Lead-Enzyme Complex Characterization

- *Task 3.1 – Production and characterization of guinea pig AChE*: A synthetic plasmid containing the gpAChE coding sequence (GenBank Accession Number NM_001177891.1) was purchased from Blue Heron Biotechnology and used as a template for standard molecular cloning techniques. Assembly polymerase chain reaction (PCR) reactions were carried out beginning at residue 30 (using hAChE numbering) and ending with a C-terminal truncation at residue 574 to remove the tetramerization domain. Additional primers appended the N-terminal CD33 leader peptide, a hexa-histadine tag and a tobacco etch virus protease site (Table S1). Fully assembled constructs were sub-cloned into the Gateway entry vector pENTR/D-TOPO™ (Life Technologies) using the TOPO technique and lambda recombined (LR) into the Gateway expression vector pT-Rex-DEST30, making the plasmid pgpAChE30-Dest. An E-Gel® Agarose Gel Electrophoresis system (Life Technologies) was used to analyze DNA samples using either 1.2% or 0.8% CloneWell with SYBR Safe™ precast E-Gel cassettes (Life Technologies). The pgpAChE30-Dest vector was linearized by restriction digest between the f1 ori and SV40 ori using Acc65I (New England Biolabs). The expression vector was then transfected into Human Embryonic Kidney (HEK)-293 cells using Lipofectamine 2000 (Life Technologies) and OptiMem (Life Technologies) according to the manufacturer's protocol. To generate a stable HEK-293 cell line, positive clones were selected over 2 weeks with Geneticin (Gibco). Constitutively expressed recombinant gpAChE was purified by immobilized nickel affinity and size exclusion chromatographies on an ÄKTAexplorer system (GE Healthcare) using a HiPrep 26/10 Desalt, a 1 mL HisTrap, a Superdex 10/300 GL and 2 HiTrap 5 mL desalt columns. Binding buffer consisted of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma), 500 mM sodium chloride (NaCl, Fisher), pH 7.6, 40 mM imidazole (Sigma) and elution buffer consisted of binding buffer in 500 mM imidazole. Storage buffer consisted of 10 mM HEPES, pH 7.0 and 100 mM NaCl. Both high and low molecular weight gel filtration kits (GE Healthcare) were used to calibrate the Superdex 10/300 GL column and assisted in determining the protein oligomerization state. This enzyme was characterized in the context of activity and binding to all lead compounds and investigated for structural characterizations.

- *Task 3.2 – Structural biological investigations of binding modes of leads to AChE targets*: X-ray crystallography and nuclear magnetic resonance (NMR) have usually been limited to lead optimization stages of drug development. We proposed utilizing these techniques early on in the drug discovery process, in a high throughput manner, to confirm binding sites for traditional HTS hits and to guide the elaboration of the fragment hits into larger molecular weight compounds that might be useful leads for drug discovery. Both the NMR and X-Ray crystallography studies were conducted at our collaborating institution, New York Structural Biology Consortium (NYSBC). While x-ray crystallography is a well-established technique within the drug discovery pipeline for its high resolution images of drug-target interactions, NMR is an additional method for investigating complexes difficult to obtain in the rigid crystal lattice. Due to the allosteric mode of binding of our leads, the crystal rigidity may be incompatible with lead binding, and we therefore proposed both structural approaches.

Task 4: In-Vitro Cell Culture Efficacy Screening and ADMET Evaluation

Once leads had confirmed protective capacity against chemical agents, these leads were down-selected for early phase ADME and cell-based toxicity studies. Often the most difficult portion of an early drug discovery campaign is achieving a balance between activity and favorable ADMET properties so that a lead compound can move into *in vivo* efficacy/toxicology studies. Generating these toxicity data in the key cell types allowed one to make early and rapid predictions about potential safety issues in drug leads.

- *Task 4.1 – Cell culture models for protection against OP-induced AChE inhibition*: Historically, cell culture models for AChE activity and inhibition have been conducted using the Ellman assay on neuroblastoma cell lines. We performed this method, as well as incorporated a novel method developed at US Army Medical Research Institute of Chemical Defense (USAMRICD) to observe neuronal signaling in networked population of neurons (Hubbard *et al*., 2012). By exploiting differentiated neuroblastoma cells and networked cholinergic neurons, our data provides more accurate experimental estimates for transitioning to *in vivo* assays.

- *Task 4.2 – Cell-based pre-clinical lead candidate toxicity studies*: Using commercially available assays, the toxicity of lead chemicals in liver, kidney and cardiac cell lines were investigated. Cytotoxicity was evaluated by monitoring the viability, as measured through metabolic activity (Promega MTS assays) of each cell line following exposure to a range of lead concentrations far in excess of the efficacious dose. In addition, to reduce the likelihood of clinical trial drug failures due to toxic effects on cellular respiration, we screened for altered cellular respiration using a plate-based, non-destructive system (Seahorse Biosciences XF Analyzer). Furthermore, selected drugs must be tested to avoid blocking the cardiac potassium channel termed hERG (Human Ether-a-go-go-Related-Gene) and this was tested using a commercially available fluorescence polarization assay. Finally, binding to serum albumins is a critical PK data point for determining the free fraction in blood, and this was measured by SPR.

Task 5: Toxicity, Pharmacokinetic/Pharmacodynamic (PK/PD) Parameter Estimates and Efficacy in Guinea Pigs

Lead candidates were chosen based on *in vitro* protective capacity, drug-like binding behavior, and cell-based toxicity results. These *in vivo* experiments at BBRC provided a rapid assessment of the therapeutic efficacy of candidates as well as began characterizing their bio-distribution and clinical toxicity effects.

- *Task 5.1 – Toxicological profiling and PK/PD monitoring using unexposed guinea pigs*: A small cohort of healthy guinea pigs was exposed to varying concentrations of lead compounds to assess lethality, tissue distribution, tissue toxicity, behavioral changes, and urinary/biliary clearance. Animals were exposed and data points obtained at multiple time points, selected from models, to determine PK and PD properties.
- *Task 5.2 – Monitoring AChE protection in guinea pigs using positron emission tomography (PET)*: Using a novel CWA derivative generated by Professor Charles Thompson at the University of Montana (Kaleem Ahmed *et al*., 2013), we proposed to monitor real-time protection against agent inhibition by PET. Published and unpublished data from the Thompson group indicates that the CWA tracer exhibits similar inhibitory effects as the V-series organophosphate agents and that the compound localizes to the brain and CNS within minutes of exposure. By pretreating with lead compounds, we hypothesized that the clearance of the tracer would be increased, thereby indicating an increase in detoxification through non-AChE inhibition.
- *Task 5.3 – In vivo challenges against nerve agent intoxication in guinea pigs*: We performed animal agent challenges using standard methods developed by USAMRICD. These agent challenges evaluated the protective ability of allosteric candidates in male and female Hartley guinea pigs against multiple agent concentrations from sub-lethal to 2-5 LD_{50} using brain and blood cholinesterase activity and survivability as our treatment readouts. It was hypothesized that pretreatment with the lead compounds would result in a shift in LD_{50} values, demonstrating the protective effect of allosteric pretreatments.

Task 6: Investigate Formulary Requirements for Oral Delivery of Therapeutic

Final development of the candidate leads was performed in conjunction with Ohio State University (OSU) formulations chemists to determine the optimal dose methods. These new CWA countermeasures will be transitioned to the Chemical Biological Medical Systems for preclinical and clinical trials.

Progress:

Screening a Hit Expansion Library for Efficacy in Human AChE

A library of 153 compounds was generated by performing a structural homology search through commercially available sources for compounds displaying chemical similarities to 9 hit structures identified in the early UC-MDI screens. As the primary lead A9 was identified in mouse enzyme studies, this compound was inserted into the screening library as a 154^{th} compound. Thus, the 154-compound library was solubilized in DMSO and aliquoted onto an Echo-certified 384-well plate for automated dilutions via a liquid handler. This instrument was used to produce a six

point dose-response curve for each compound across numerous 384-well assay plates, with each plate containing internal controls for enzyme efficacy. The enzyme activity of human AChE in the presence of each of these compounds was evaluated at all concentrations and dose-responses were characterized. Of the 154 compounds tested, 51 new compounds were identified as activating the human enzyme. Interestingly, compound A9 was not among these 51 compounds, but was found to be among a smaller set of compounds inhibiting the enzyme.

Aging has Little Effect on the Efficacy of Lead A9 in Mouse AChE

A critical aspect of all therapeutic countermeasures is stability, and for those destined for use in austere environments, heat stability is even more crucial. Therefore, the ability of compound A9 to allosterically modulate AChE activity after being stored at situationally-relevant temperatures (45°C) was evaluated. Even after being stored for multiple days at this temperature, the compound was as efficacious as freshly prepared (Figure 1). These results clearly suggest that despite the structural changes seen by NMR over the same time-period (Figure 2) the efficacy of the compound is unchanged.

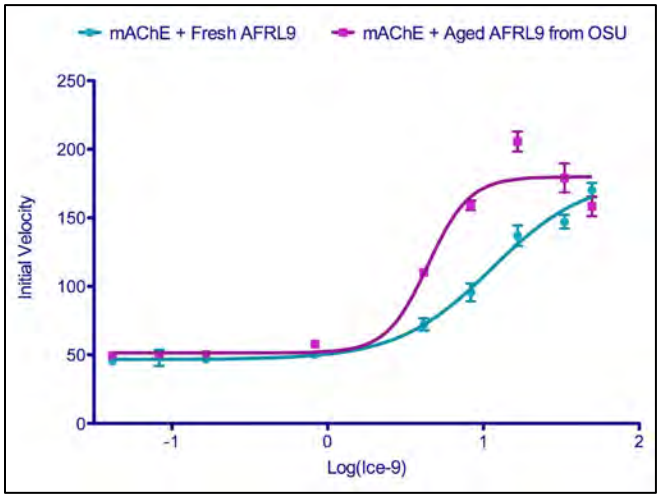

Figure 1. *Effect of heat induced aging upon A9 efficacy. The small shift in the EC_{50} value is not statistically significant ($P < 0.05$), n=3.*

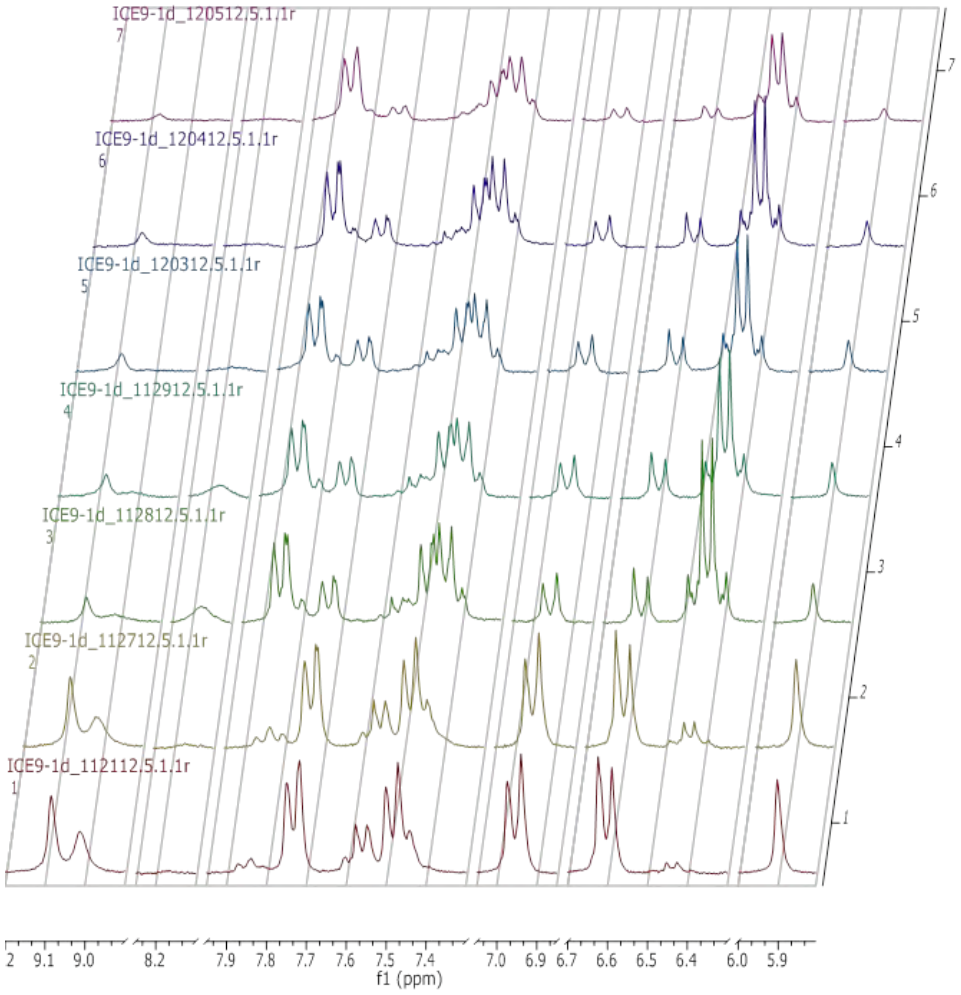

Figure 2. *Time trace of NMR showing structural changes of the freshly prepared compound.*

Enzyme Kinetics Show A9 Activates AChE by Enhancing Catalytic Efficiency

To further clarify the mode of action by which A9 is able to modulate AChE activity, detailed kinetic experiments were performed to monitor changes in catalytic parameters (Figure 3). By varying both substrate and A9 concentrations, we were able to ascertain that the catalytic efficiency was being improved, as measured by an increase in the V_{max} of the enzyme (Table 1). Importantly, the Michaelis constant (K_m) was unchanged, suggesting that the compound was interacting at a site different from the substrate binding pocket. From the EC_{50} value at a given substrate concentration (for example, 1 mM acetylthiocholine, ATC) we can calculate the dissociation constant for the A9-AChE complex. Using the example provided, we found that the K_D is approximately 1.9 µM, a concentration that is lead-like in nature and approaches can provide optimized structures in future work incorporating the observations from structural studies such as x-ray crystallography or NMR spectroscopy.

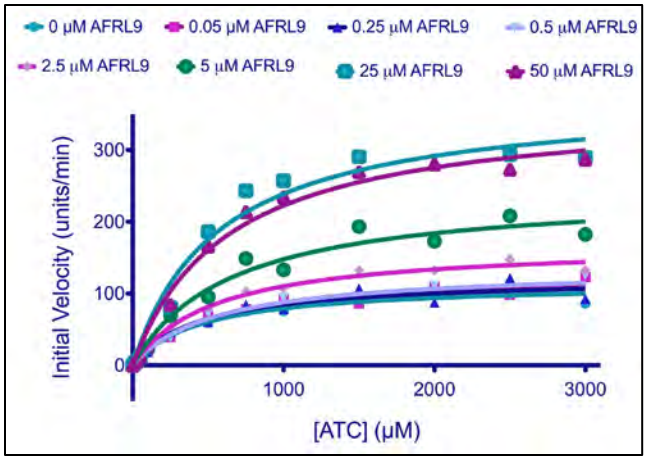

Figure 3. *Enzyme kinetic evaluation of murine AChE in the presence of A9. The K_m remains constant at 0.45 ± 0.08 mM and 0.62 ± 0.09 mM for compound-free and 50 µM A9, respectively; while the V_{max} values change from 115 ± 6 mU/min to 362 ± 17 mU/min, respectively.*

Table 1. *Kinetic Parameters Murine AChE in the Presence of Varying Levels of A9*

[AFRL9]	K_m (mM)	V_{max}
0	0.45 ± 0.08	115 ± 6
50 nM	0.53 ± 0.09	132 ± 7
250 nM	0.4 ± 0.1	122 ± 9
500 nM	0.6 ± 0.1	135 ± 7
2.5 µM	0.54 ± 0.06	169 ± 6
5 µM	0.6 ± 0.1	244 ± 17
25 µM	0.6 ± 0.1	375 ± 27
50 µM	0.62 ± 0.09	362 ± 17

Crystal Structures Do Not Show A9 Bound to Human or Mouse Enzymes

Preliminary studies involving soaking crystals of both human and mouse AChE with high levels of compound A9 were performed. Neither set of crystals resulted in observation of electron densities corresponding to A9 in the diffraction data. Co-crystallization experiments remain in the future.

Cell Culture Studies with A9 Show Low Cytotoxicity at High Levels

In parallel with detailed *in vitro* bench-top characterization of the A9 mode of action in AChE, we began evaluating the compound's ability to be tolerated by mammalian cell cultures. Microscopic observations of cell cultures exposed to varying levels of A9 for 24 hours and 96 hours revealed minor morphological changes between 2.5 and 5 µM and cell death at 25 µM (Figure 4). Further investigation into this phenomenon using a cell proliferation assay confirmed the initial observations and we were able to determine the LD_{50} value at approximately 9 µM

(Figure 5). We believe that A9 is not a drug candidate since toxicity was observed within or close to the therapeutic concentrations and more work should be done to determine a chemical homolog with an improved therapeutic index.

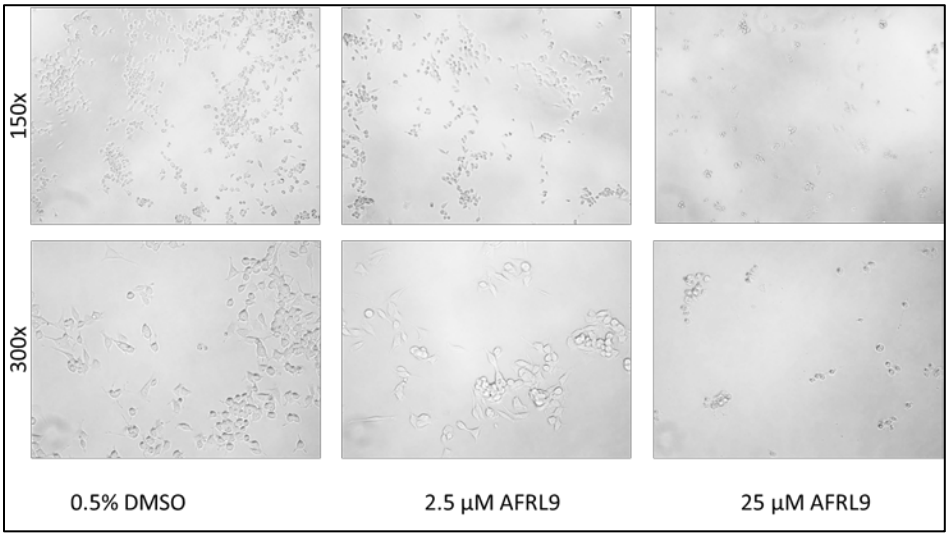

Figure 4. *Microscopic evaluation of the cytotoxic effect of A9 on Neuro2A cell cultures after 24 hours. Cells exhibit moderate but clear morphological changes at 2.5 µM and by 25 µM are exhibiting signs of cell death.*

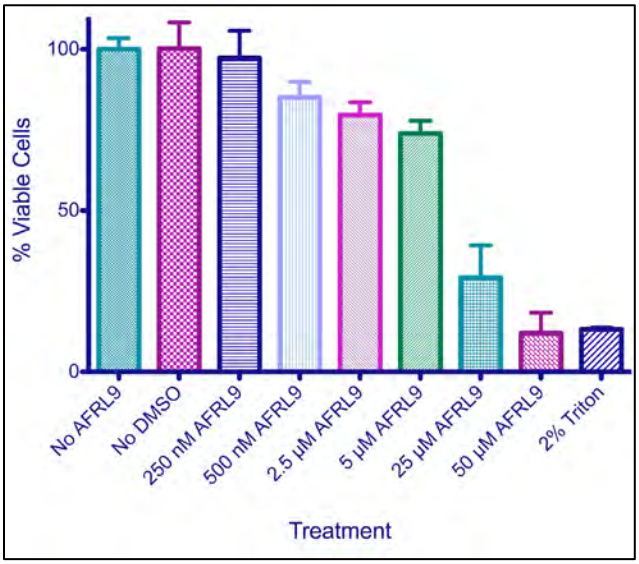

Figure 5. *Cytotoxicity of A9 in Neuro2A cells at 24 hours as measured in quadruplicate by a cell viability assay. Results confirm morphological changes observed by microscopic analyses. A Dunnett's multiple comparison test showed that the 25 and 50 µM treatments were significantly different than the "no AFRL9" control ($p < 0.05$).*

Discussion/Conclusions:

The high efficacy of lead compound A9 even after being exposed to elevated temperatures is a positive result suggesting that, although the compound may have breakdown degradation products that must be identified, the predominant compound present after aging is the pharmaceutically active moiety. This high temperature stability is critical when considering that the environments potentially identified as the deployment destinations for this pre-treatment therapeutic are likely to be subject to extreme temperatures and have limited access to long-term refrigeration and climate control. Our current work has demonstrated that A9 is an activator of mouse AChE, but at this time we do not have data on A9 activation of human AChE. However, we are hopeful that studies testing the protective effect of A9 on the human enzyme will show similar efficacy. In the event that such efficacy is not observed, lead-optimization could be pursued through combinatorial chemistry partnerships. We theorize that a reason for altered activity between the two enzymes may lie in the C-terminal tail of the human enzyme that is not present in the mouse variant. This tail may be covering a region critical to the activity of the compound. Additionally, although our kinetic observations do not conclusively show that the compound functions through an allosteric mechanism, combined with the knowledge of the structural rigidity surrounding the active site, these data are highly suggestive of such a mechanism.

Furthermore, the lack of observation of binding in the crystal structure is not conclusive of non-binding to either enzyme. It is frequently seen that small molecules are capable of modulating enzyme activity and yet cannot be bound in a crystal. A brief, non-exhaustive list of reasons for these results include rapid, transient binding; occlusion of the binding site by crystal contacts; lack of solvent channels in the vicinity of the binding site; and occupation of the binding site by crystallographically important ligands such as buffers, salts or precipitants.

This compound may be a valid neuroprotectant for short-term pretreatments and our initial cell culture cytotoxicity tests demonstrate promising results. We anticipate evaluating cytotoxic effects in liver, kidney and gut cells, as well as determining the gut absorption parameters. These tests will become standard evaluations to be performed in parallel with bench-top tests for each lead as it advances. By executing cell culture-based ADMET and mode-of-action studies in parallel, we expect to greatly reduce the time from hit to lead to viable therapeutic candidate and begin collecting animal exposure data sooner. The end result of all of this will be getting a therapeutic countermeasure for preventing chemical agent intoxication to the warfighter sooner.

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