



Research Article

Development of a High-throughput Assay to Identify Novel Reactivators of Acetylcholinesterase

Becky L Hood^{1*} , Stefan Vasile¹, Gideon Shapiro² and James D Talton¹

¹Alchem Laboratories Corp., Alachua, FL, USA

²Pharmorquest Inc, USA

Received: 22 August, 2025

Accepted: 19 September, 2025

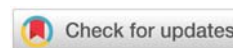
Published: 20 September, 2025

***Corresponding author:** Becky L Hood, Alchem Laboratories Corp., Alachua, FL, USA, E-mail: bhood@alchem.com

Keywords: Acetylcholinesterase; Reactivator; Organophosphate; Nitrophenyl isopropyl methylphosphonate; NIMP; Amplex Red

Copyright License: © 2025 Hood BL, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

<https://www.chemisgroup.us>



Abstract

Acetylcholinesterase (AChE) is a key enzyme in the nervous system capable of breaking down acetylcholine (ACh). ACh is a critical neurotransmitter that signals between nerve cells and muscles. Organophosphates (OPs), used as insecticides and nerve agents, irreversibly inhibit AChE by forming a covalent bond with the enzyme's active site. Since exposure to these nerve agents can quickly lead to death, many researchers have strived to discover new drugs that will reverse the inhibition of AChE by such agents. The goal was to develop a high-throughput miniaturized assay for testing large numbers of compounds for AChE reactivation quickly and reliably. Here, we describe our miniaturized assay that utilizes the sarin surrogate nitrophenyl isopropyl methylphosphonate (NIMP) in an Amplex Red coupled assay and its validation with 42 compounds selected from an *in silico* virtual screen.

Introduction

Organophosphorus nerve agents such as isopropyl methylphosphonofluoridate (sarin) and S-2-diisopropylaminoethyl O-ethyl methylphosphonothioate (VX) are potent acetylcholinesterase inhibitors. Exposure to these agents can rapidly lead to the accumulation of the neurotransmitter acetylcholine in neuromuscular junctions and cholinergic synapses [1]. These reactive organophosphates inhibit the hydrolytic action of acetylcholinesterase (AChE) by phosphorylating the serine of AChE's active site [2]. Phosphorylation is persistent because spontaneous reactivation is slow [3]. If left untreated, organophosphate (OP) poisoning leads to dose-dependent toxic signs including tremor, hypersecretion, seizures, respiratory depression and eventually death. Various quaternary oxime reactivators such as Pralidoxime (2-PAM) [4] and obidoxime [5], combined with anticholinergics such as atropine and scopolamine, have been used to treat acute poisoning by OP nerve agents, while seizures are often controlled by the administration of GABA receptor agonists such as diazepam or midazolam [6].

Organophosphorus nerve agents pose a special threat to the military, including active-duty combat personnel, nerve agent stockpile handlers, and nerve agent demilitarisation workers [7].

Cholinesterase reactivators are generally used as antidotal therapies for pesticides and nerve agent poisoning [8]. The discovery of 2-PAM as a reactivator of OP inactivated AChE [9] led to numerous mono- and bis-quaternary oximes AChE reactivators with activity *in vitro* and *in vivo*. 2-PAM, obidoxime [10], and asoxime chloride (HI-6) [11] were fielded as OP nerve agent antidotes for combat soldiers around the world [12]. However, quaternary oximes are not able to cross the blood-brain barrier (BBB), thus limiting their therapeutic efficacy. Therefore, it is of great interest to identify novel reactivators that are brain-permeable. To this goal, Amitai et. al. [13] discovered four novel reactivators of both sarin- and VX-inhibited butyrylcholinesterase (BChE), which also directly detoxified Sarin. These authors expect that their new reactivators will be permeable to the BBB. To date, compounds with broad-spectrum reactivation are limited. Identifying classes of compounds to fill the gaps would be very beneficial.

The assay that is described here for identifying novel reactivators of OP inactivated AChE utilises the sarin surrogate NIMP, which was designed to phosphorylate cholinesterases with the same moiety as sarin, making it highly relevant for the study of cholinesterase reactivators [14,15]. NIMP is a potent inhibitor of rat brain, skeletal muscle, diaphragm, and serum AChE, as well as human erythrocyte AChE and serum BChE in vitro. Although safer to use than sarin and other nerve agents, keeping the exposure to a minimum is desired. Having a low-volume assay using nanoliter quantities of NIMP was our goal for safety reasons as well as for cost savings.

For this assay, we chose to use an Amplex Red fluorescent assay to measure the acetylcholinesterase activity. It is based on the coupled Amplex Red fluorescent assay available commercially from Invitrogen (brand of Fisher Scientific) [16,17], which is more sensitive than the Ellman's assay. Unlike the Ellman's assay, compounds with sulfur do not interfere with the Amplex Red assay [18]. An additional advantage of the Amplex Red assay is that it uses the natural substrate (acetylcholine), whereas the Ellman's assay uses acetylthiocholine. However, the Amplex Red assay is not a perfect assay because some fluorescent compounds or fluorescent quenchers will interfere with the fluorescent signal of the Amplex Red assay. The issue with fluorescent compounds appearing as reactivators is overcome by taking multiple time points. A fluorescent compound will not increase its fluorescence with time, whereas a true reactivator will increase the fluorescent signal with time as the substrate is turned over by enzyme activity. Also, quenching (such as 12.5 μ M HI-6, see Figure 6) will eventually be overcome by the enzyme activity at later time points. We see this in the dose response curves (DRCs) HI-6. The curves become well-behaved S curves at later time points in the assay. Figure 1 shows the coupled reaction involved in this assay.

Materials and methods

Reagents and instruments

The sarin surrogate isopropyl (4-nitrophenyl) methylphosphonate (NIMP, cat # SY247222) was purchased from Acello Chembio (San Diego, CA). Amplex Red reagent (cat # A12222) was purchased from Thermo Fisher Scientific (Waltham, Massachusetts). Horseradish peroxidase (cat #

P8375), Choline Oxidase (cat # C5896), acetylcholine chloride (cat # A2661), human recombinant acetylcholinesterase (cat # C1682), HI-6 (cat # SML0024), 2-PAM (cat # 1553000), dimethyl sulfoxide (DMSO, cat # D2650) and Tris-HCl (cat # T3253) were purchased from Sigma Aldridge (Saint Louis, MO). 1536-well plates were purchased from Greiner (cat # 789888).

NIMP may cause skin irritation, serious eye irritation and respiratory irritation. Alchem protocols consistent with safety and biosafety policies, as well as EPA and Biohazardous material requirements, were followed while working with NIMP.

Stocks of the reagents were made as follows: The Sigma acetylcholinesterase is expressed in human HEK 293 cells as a 583 amino acid glycoprotein with a calculated molecular mass of 64.6 kDa. It is supplied as a powder lyophilized from a 0.22 μ m filtered solution in PBS, pH 7.4. A stock of acetylcholinesterase (167U/ml) was prepared in cold 1X assay buffer, and aliquots were stored at -80°C . One unit is defined as the amount of enzyme that will hydrolyse 1.0 micromole of acetylcholine to choline and acetate per minute at pH 8.0 at 37°C . Amplex Red was made in DMSO at 5 mg/ml. Aliquots were frozen at -20°C and stored for no longer than 2 weeks at a time. A stock of 5X assay buffer (250mM Tris, pH 8) was filtered through a 0.2 μ m filter and stored at 4°C . On the day of the assay, the 5X buffer was diluted with sterile ultrapure water to 1X (50mM Tris, pH 8) and warmed to room temperature before beginning the assay. A stock of horseradish peroxidase was made in cold 1X assay buffer at 200U/ml. Aliquots were frozen at -80°C . A stock of choline oxidase was made in cold 1X assay buffer at 20U/ml, and aliquots were frozen at -80°C . A stock of 100mM acetylcholine chloride was made in sterile ultrapure water, aliquoted and stored at -20°C . NIMP was made fresh in DMSO for every set of experiments.

Equipment used for assay development included: a Labcyte/Beckman Coulter Echo 555 Acoustic transfer system (San Jose, CA), a Beckman Coulter BioRAPTR FRD (Brea, CA), a Perkin Elmer Envision multi-mode plate reader (Waltham, MA) and an Eppendorf 5810 centrifuge (Hamburg, Germany).

Basic assay protocol

NIMP is dispensed to a Greiner 1536 well plate using a Labcyte Echo 555 Acoustic transfer system. AChE is then dispensed to the plate using a Beckman Coulter BioRAPTR FRD. The plate is centrifuged in an Eppendorf 5810 centrifuge. Test compounds and control reactivators are dispensed to the plate with a Labcyte Echo 555 dispenser. The substrate mixture is dispensed to the plate using a Beckman Coulter BioRAPTR FRD, and the plate is centrifuged with an Eppendorf 5810 centrifuge. The plate is then incubated at room temperature in the dark. Finally, the plate is read on a Perkin Elmer Envision multi-mode plate reader (Ex 555 and Em 590). Specific conditions will be presented in the results section.

Data analysis

Assay development data were analysed using GraphPad Prism (GraphPad Software, Boston, MA). The formula for per cent reactivation is:

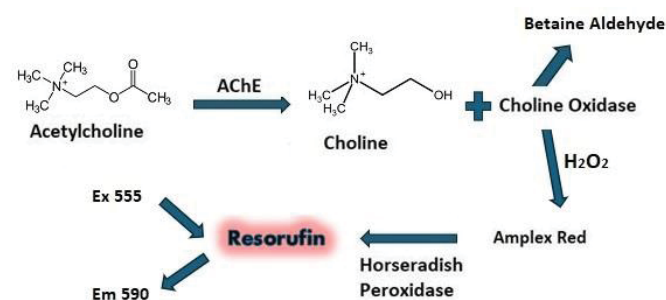


Figure 1: Amplex Red Assay Reaction. Acetylcholinesterase hydrolyses acetylcholine to choline. Choline is then oxidised by choline oxidase, producing betaine aldehyde and H_2O_2 . H_2O_2 reacts with Amplex Red, catalysed by HRP, producing resorufin, which can be quantified by fluorescence.

$$[(\text{test value} - \text{average Min value}) / (\text{average Max value} - \text{average Min value})] \times 100$$

Min value (Negative Controls) wells containing AChE + Substrate + NIMP + DMSO.

Max value (Positive Control control-wells with AChE + Substrate + DMSO

Test wells contain AChE + Substrate + NIMP + Reactivator
Test compounds

Positive control compounds are HI-6 and 2-PAM

It should be emphasised that all wells contain the same percentage of DMSO. Reactivators are diluted in DMSO. NIMP is diluted in DMSO. Thus, when NIMP is added to the plates, 10nl of DMSO is added to the Max control wells. When reactivators are added to the plate, 10nl of DMSO is added to the Min control wells and Max control wells. When whole plates were run, the percent reactivation, Z-score values and IC₅₀ values were determined using CBIS software (ChemInnovation Software, Inc., San Diego, CA).

Z-score calculation for quality control

A statistical parameter has been developed which helps to determine if an assay is robust. This is known as the Z-factor or Z'. The Z-factor is established from four parameters: the means (μ) and standard deviations (σ) of both the positive (p) and negative (n) controls (μ_p , σ_p , and μ_n , σ_n) [19]. Z-factor is computed as:

$$\text{Z-factor} = 1 - (3 \cdot \sigma_p + 3 \cdot \sigma_n) / [\mu_p - \mu_n]$$

Z' factor between 0.5 and 1.0 has been shown to represent a robust and reliable assay.

Results: Optimization and Miniaturization of the AChE Reactivation Assay

Acetylcholinesterase 1536 well assay development

Before initiating the development of the reactivator assay, we had previously developed an acetylcholinesterase inhibitor assay using the Amplex Red coupled assay. The assay conditions for the inhibitor assay were incorporated into the reactivator assay. The following processes and conditions were tested during the development of the 1536-well acetylcholinesterase inhibitor assay:

Titration and time course of acetylcholinesterase

The amount of acetylcholinesterase used in the assay was determined with the goal of using as little enzyme as possible but with a quantity that would provide a robust and reproducible signal. A range of AChE concentrations from 0.025 to 0.25 units/ml was tested in the assay. It was determined that 0.025 U/ml was a suitable concentration for the assay (see Figure 2A).

Acetylcholine titration

For a biochemical assay, it is important to determine the K_m for the substrate (assuming Michaelis–Menten kinetics;

K_m is the concentration of substrate which permits the enzyme to achieve half V_{max}). Typically, the enzyme assay is run with the substrate at the K_m. Because this assay is coupled, using 3 separate enzymes (AChE, choline oxidase and HRP), we will only be able to calculate the apparent K_m for acetylcholine. Horseradish peroxidase and choline oxidase are not limited, and the concentrations are set at concentrations used in commercial kits for this Amplex Red coupled assay (final assay concentrations are 1 U/ml Horseradish Peroxidase and 0.1 U/ml choline oxidase). The assay is run at a fixed AChE concentration (0.25 U/ml) while varying the concentration of acetylcholine. It was determined that the apparent K_m for acetylcholine in the assay is 14 mM. Data is shown in Panel B of Figure 2.

Acetylcholinesterase reactivation assay development

Initial data generated for HI-6 and 2-PAM

As previously stated, the initial assay conditions for the reactivation assay were based on the acetylcholinesterase inhibition assay. Initially, a 4 μ l assay, which followed the following steps, was attempted: First, dilutions of NIMP were prepared in DMSO. Then, either 10 nl of the NIMP dilutions or 10 nl of DMSO for the positive controls were transferred to a Greiner 1536 well assay plate using a Labcyte/Beckman Coulter Echo 555 acoustic dispenser. Then, 2 μ l of AChE (Final Assay Concentration (FAC) 0.025 U/ml) was dispensed using a Beckman Coulter BioRAPTR FRD. Next, the plate was centrifuged at 1000 rpm for 1 minute and incubated at room temperature for 30 minutes. During the incubation, dilutions of the reactivators (2-PAM and HI-6) were made in DMSO and transferred to an ECHO-qualified 1536-well plate (in triplicate). After the 30-minute incubation, 10 nl of the reactivators was transferred from the ECHO source plate to the assay plate using a Labcyte/Beckman Coulter Echo 555 acoustic dispenser. Immediately, 2 μ l of substrate mixture was added (final concentrations were 15 μ M acetylcholine chloride, 200 μ M Amplex Red, 0.1 U/ml Choline Oxidase and 1 U/ml horseradish peroxidase). The plate was centrifuged at 1000 rpm for 1 minute and then read on a Perkin Elmer Envision (excitation 555 and emission 590)

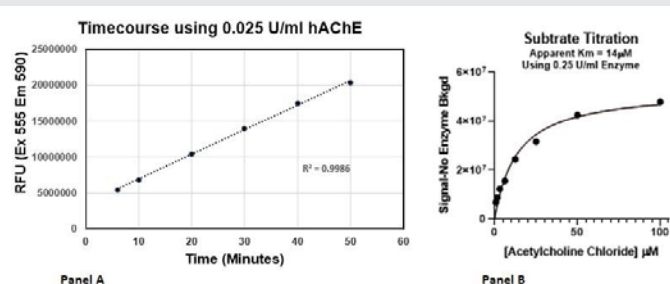


Figure 2: Acetylcholinesterase Time Course and Acetylcholine Titration. Panel A shows the timecourse using 0.025 U/ml of human AChE in a 4 μ l assay. 2 μ l of enzyme mixture (2 U/ml horseradish peroxidase, 0.2 U/ml choline oxidase and various concentrations of AChE in 50mM Tris pH 8.0) was added to the wells. 2 μ l of substrate/Amplex Red mixture (30 μ M acetylcholine chloride and 400 μ M Amplex Red in 50 mM Tris pH 8.0) was added to the wells. The plate was incubated in the dark at room temperature, and the fluorescence was read at various time points at ex 555 and em 590. Panel B shows the acetylcholine chloride titration in the 4 μ l assay with conditions described in panel A. In this assay, 0.25 U/ml of hAChE was used.

every 10 minutes. Very weak activity was observed by 2-PAM and approximately 50% reactivation with HI-6. Because 2-PAM was a weak reactivator compared to HI-6, HI-6 was used for subsequent assay development experiments. Initial Data is shown in Supplemental Figures 1 and 2. This data was generated with FAC of NIMP set at 0.059 nM.

DMSO tolerance

For the acetylcholinesterase inhibitor assay, the dimethylsulfoxide (DMSO) exposure is not only from the test compounds but also from the NIMP. When titrated in the inhibitor assay, DMSO had a slight effect above 0.25% (Supplement Figure 3). However, in the AChE reactivator assay, the enzyme is first exposed to NIMP, which is solubilised in DMSO, and when the reactivator test compounds are added, these are also solubilised in DMSO, which doubles the DMSO concentration. Plus, we believe there is extra stress on the enzyme from the phosphorylation by NIMP, which might reduce the enzyme's tolerance to the DMSO. With these considerations in mind, it was decided to measure the AChE activity in a 4 μ l and an 8 μ l assay. To do this, the enzyme was exposed to varying concentrations of DMSO delivered to the plates with the Labcyte/Beckman Coulter Echo 555 acoustic dispenser. Choline oxidase and horseradish peroxidase are added with the substrate and Amplex Red (FAC:15 μ M acetylcholine chloride, 1 U/ml Horseradish Peroxidase, 0.1 U/ml choline oxidase, 200 μ M Amplex Red in 50 mM Tris, pH 8.0). This method of addition was used for all subsequent experiments. The data shown in Figure 3 demonstrates that the enzyme functions better in the 8 μ l assay with the optimal DMSO concentration no higher than 0.25%. Thus, it was decided that the assay would be an 8 μ l assay using 10 nM of NIMP and 10 nM of test compound (reactivators).

NIMP Titration in 8 μ l AChE reactivation assay

Once it was confirmed that the AChE activity improved in the 8 μ l assay, the next step was to test NIMP in the 8 μ l assay. Dilutions of NIMP were made in DMSO, and 10 nM of each dilution was transferred to the assay plate using an ECHO 555 acoustic dispenser, and 4 μ l AChE was dispensed using a BioRAPTR FRD. The plate was centrifuged for 1 minute at 1000 rpm, and 4 μ l substrate mixture (FAC:15 μ M acetylcholine chloride, 1 U/ml Horseradish Peroxidase, 0.1 U/ml choline oxidase, 200 μ M Amplex Red in 50 mM Tris, pH 8.0) was added to the plate. The plate was centrifuged and incubated in the dark at room temperature for 30 minutes. The plate was read on a Perkin Elmer Envision with Ex 555 and Em 590. Because it is easy to completely kill the enzyme with NIMP, we wanted to work at a NIMP concentration just above baseline (EC₇₅). Based on NIMP dose response curves, the concentration for NIMP that gives EC₇₅ was determined to be 0.09 nM (data shown in Figure 4).

HI-6 Titration

The control reactivator (HI-6) was titrated to determine the best concentration for the assay. For this experiment, NIMP was dispensed at a final concentration of 0.09 nM (10 nM)

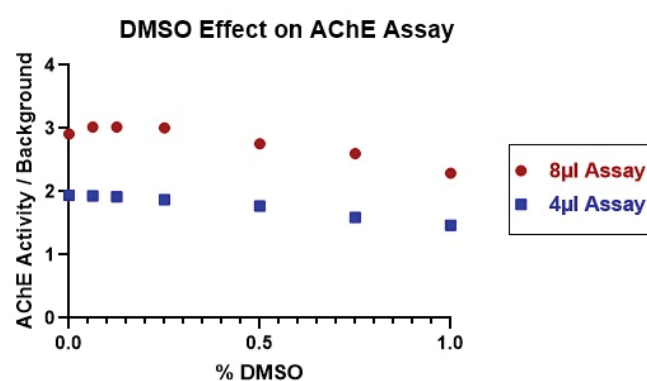


Figure 3: DMSO Effect on AChE Activity. Varying amounts of DMSO were dispensed to 2 assay plates using the ECHO 555 acoustic dispenser. Then, 2 μ l of AChE enzyme (final concentration 0.025 U/ml) was dispensed to the top 16 rows and 2 μ l of buffer was dispensed to the bottom 16 rows of plate 1. Likewise, 4 μ l of enzyme was dispensed to the top 16 rows and 4 μ l of buffer was dispensed to the bottom 16 rows of plate 2. The two plates were centrifuged for 1 minute at 1000 rpm. Then, 2 μ l substrate was dispensed to all wells of plate 1, and 4 μ l was dispensed to all wells of plate 2. The plates were centrifuged again and incubated for 30 minutes at room temperature in the dark. Then the plates were read on the Envision at Ex 555 and Em 590.

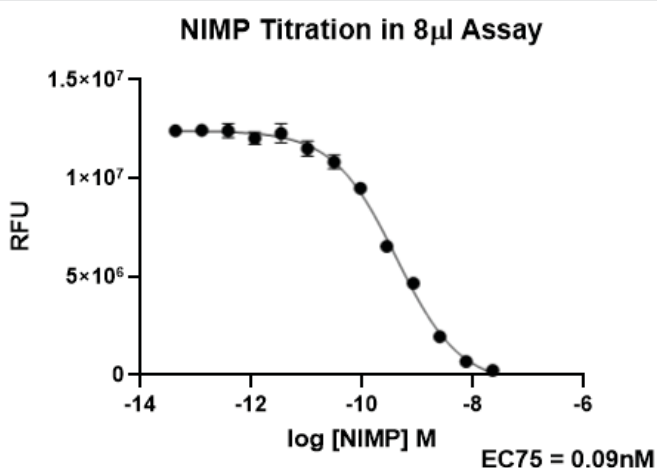


Figure 4: EC₇₅ of NIMP in the 8 μ l AChE Reactivation Assay. NIMP was diluted in DMSO and transferred to an assay plate using the ECHO 555 acoustic dispenser. Then, 4 μ l of AChE enzyme (final concentration 0.025 U/ml) was dispensed to wells. The plate was centrifuged for 1 minute at 1000 rpm. Next, 4 μ l substrate mixture (FAC:15 μ M acetylcholine chloride, 1 U/ml Horseradish Peroxidase, 0.1 U/ml choline oxidase, 200 μ M Amplex Red in 50 mM Tris, pH 8.0) was dispensed to the plate. The plate was centrifuged and incubated for 30 minutes at room temperature in the dark and read on the Envision at Ex 555 and Em 590. Data were fitted with a four-point logistical model in GraphPad Prism.

using the ECHO 555. Then 4 μ l of AChE (final concentration 0.025 U/ml) was dispensed with a Beckman Coulter BioRAPTR. The plate was centrifuged at 1000 rpm for 1 minute, and immediately, 10 nM of HI-6 diluted in DMSO at various final concentrations was dispensed to the plate using a Labcyte Echo 555. The plate was centrifuged again at 1000 rpm for 1 minute and incubated for 15 minutes at room temperature. Then 4 μ l of the substrate mixture (FAC:15 μ M acetylcholine chloride, 1 U/ml Horseradish Peroxidase, 0.1 U/ml choline oxidase, 200 μ M Amplex Red in 50 mM Tris, pH 8.0) was dispensed to the plate. The plate was centrifuged at 1000 rpm for 1 minute, then read on a Perkin Elmer Envision every 10 minutes for a total of 90

minutes. The data (shown in Figure 5) indicate that 6.25 μM HI-6 is the optimal concentration for the assay because 12.5 μM HI-6 quenches the signal. HI-6 at 6.25 μM yields roughly 73% reactivation at 90 minutes in this experiment.

Final optimization of the AChE reactivation assay

When testing compounds in the assay, it was discovered that incorporating a titration of the NIMP before each experiment proved beneficial. The goal was to refine the NIMP concentration to get 50% reactivation by 6.25 μM HI-6 in 30 minutes. In this way, variation caused by NIMP, which is a compound that is easily oxidised and will readily lose activity upon oxidation, could be controlled. With that said, generally, the deviation from 0.09 nM NIMP is only a modest shift from experiment to experiment. A time course for the assay was also incorporated, taking readings every 30 minutes up to 5 hours. By doing this, fluorescent artifacts are identified because their fluorescence will not increase with time. Also, fluorescent quenchers have a diminished effect at 5 hours as the enzyme converts the substrate. In every experiment, we include a dose response of the two control compounds (HI-6 and 2-PAM). At the 5-hour time point, the EC₅₀ is calculated for each compound using the CBIS software. Shown in Figure 6 are representative curves of both 2-PAM and HI-6 in our assay at the 5-hour time point and the average EC₅₀ values for all our experiments to date.

Final acetylcholinesterase reactivation assay protocol

Based on the initial developmental studies described above, the following protocol has been developed.

1. Titrate the NIMP so that the concentration yields 50% Reactivation by 6.25 μM HI-6 at 30 minutes.
2. Dispense 10 nl of NIMP or DMSO to a 1536-well Greiner assay plate. The NIMP is dispensed into all columns except for columns 3 and 4. DMSO is dispensed into

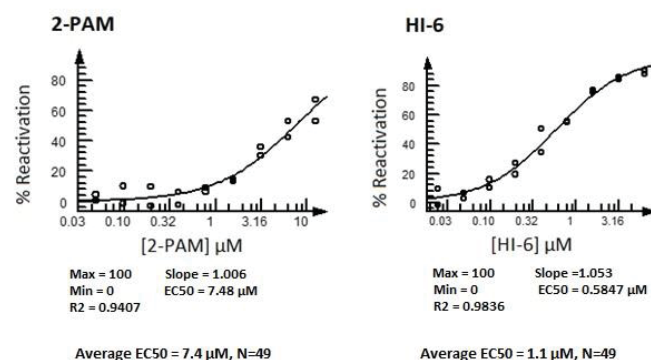


Figure 6: Dose Responses of 2-PAM and HI-6 in AChE Reactivation Assay. The 5-hour data was analysed using the CBIS software. The average values shown at the bottom of the figure were from 49 individual experiments.

columns 3 and 4, which are the 100% enzyme activity control wells.

3. Dispense 4 μl of human acetylcholinesterase (FAC are 0.025 U/ml) to all wells of the plate using the BioRAPTR FRD.
4. Centrifuge the plate for 1 minute at 1000 rpm in an Eppendorf centrifuge.
5. Immediately dispense 10 nl test and control reactivators to the plate (DRCs of control reactivators 2-PAM and HI-6 are included). Each test compound is run in duplicate with 9 dilution points. Dispense 10 nl of DMSO into columns 1, which are the 0% reactivation controls and columns 3 and 4 (again, these are 100% enzyme activity control wells). Dispense 10 nl of HI-6 to column 2 (FAC 6.25 μM) for the reactivation control.
6. Dispense 4 μl of substrate mixture (FAC:15 μM acetylcholine chloride, 1 U/ml Horseradish Peroxidase, 0.1 U/ml choline oxidase, 200 μM Amplex Red in 50 mM Tris, pH 8.0) to all wells of the assay plate using a BioRAPTR FRD.
7. Centrifuge the plate for 1 minute at 1000 rpm in an Eppendorf centrifuge.
8. Read the plate on a Perkin Elmer Envision (Ex 555 and Em 590). For screening compounds at a single concentration, two readings are taken at 30 minutes and 5 hours. If the percent reactivation does not change, the compound is a fluorescent artifact. For dose response curves, readings are taken every 30 minutes to evaluate the speed at which the reactivation occurs. The EC₅₀ curve is generated using the 5-hour time point.

Using this protocol, good plate statistics were consistently obtained, with an average Z value of 0.72 and an average signal window of 5.6-fold. Average CV is 7.4%.

Validation with compounds from an In Silico virtual screen

To validate the AChE reactivation assay, we conducted an in

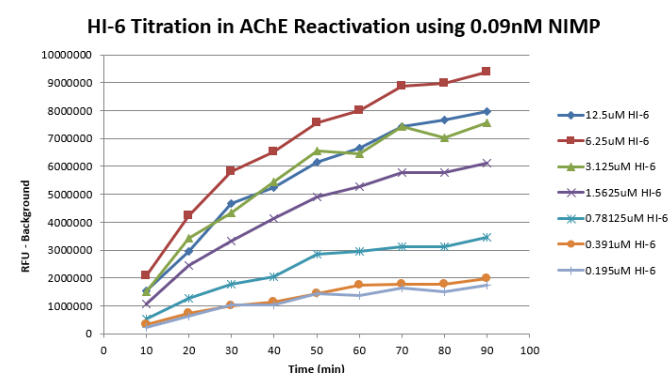


Figure 5: HI-6 Titration in AChE Reactivation Assay: Dispensed NIMP (FAC 0.09 nM) to the assay plate using a Labcyte Echo 555. Then, dispensed 4 μl of AChE enzyme using a Beckman Coulter BioRAPTR. The plate was centrifuged at 1000rpm for 1 minute, and immediately, 10 nl of HI-6 at various concentrations was dispensed to the plate using a Labcyte Echo 555. The plate was centrifuged again at 1000 rpm for 1 minute and incubated for 15 minutes at room temperature. Then, 4 μl substrate mixture (FAC:15 μM acetylcholine chloride, 1 U/ml Horseradish Peroxidase, 0.1 U/ml choline oxidase, 200 μM Amplex Red in 50 mM Tris, pH 8.0) was added to the plate. The plate was centrifuged at 1000 rpm for 1 minute and then read on a Perkin Elmer Envision every 10 minutes.

silico screen of 20,000 oxime compounds from the Chemspace oxime small molecule library using an oxime reactivator docking model to rank candidates for assay validation. The human homology oxime reactivator docking model was developed in collaboration with Molecular Forecaster Inc. The model, which will be described in detail elsewhere, was based on the mouse sarin-inactivated AChE crystal structure (PDB ID: 2WHP) complexed with the oxime reactivator HI-6. The 2WHP structure is unique in having the oxime oxygen function of the oxime in the correct directional alignment to mediate reactivation by nucleophilic attack on the phosphorus atom of the phosphorylated active site AChE-OP serine residue (see Figure 7 below). The reactivator docking model was optimised from the X-ray structure using a molecular dynamics approach, simulating the oxime attack on phosphorus. A set of 42 oximes was selected from the 20,000 oxime compound library screen based on high docking score ranking, chemical diversity and commercial availability.

From the NIMP assay screen of the 42 in silico selected compounds, hit oxime reactivator Compound 8 was identified

with an average $EC_{50} = 4.1 \mu M$ ($N=3$). The data for this validation screen of the 42 compounds is shown in Figure 8. Figure 9 shows the dose curves for Compound 8 in the 3 assays. The

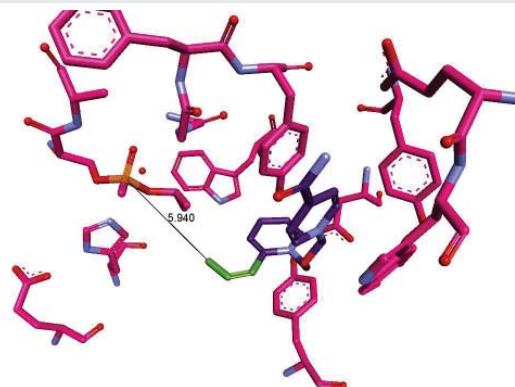


Figure 7: PDB Crystal Structure 2-WHP, Mouse AChE-OP derived from Sarin in "productive" Complex with HI-6 Reactivator. The HI-6 oxime oxygen (green) is oriented toward the phosphorus of AChE-OP at a distance of 5.9 angstroms.

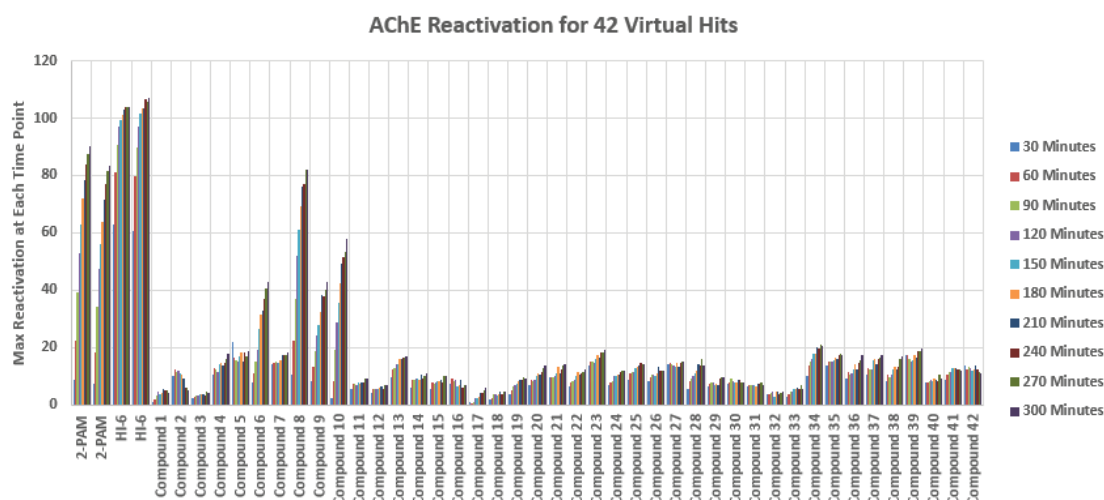


Figure 8: Results of Validation Screen for the AChE Reactivation Assay. An *in silico* virtual screen was performed using a library of 20,000 oxime small molecules. Forty-two compounds were tested in this experiment in a dose-response using the protocol described in the "Final Acetylcholinesterase Reactivation Assay Protocol" section.

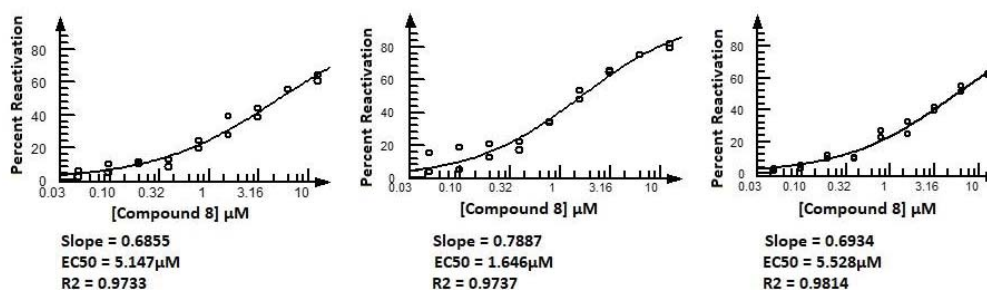


Figure 9: Dose Response Curves for Compound 8 in the AChE Reactivation Assay. Dispensed NIMP (FAC 0.09nM) to the assay plate using a Labcyte Echo 555. Then, dispensed 4 μL of AChE enzyme using a Beckman Coulter BioRAPTR. The plate was centrifuged at 1000rpm for 1 minute, and immediately, 10 μL of test compounds, along with control reactivators, were dispensed into the assay plate using a Labcyte Echo 555. Then, 4 μL substrate mixture (FAC:15 μM acetylcholine chloride, 1 U/ml Horseradish Peroxidase, 0.1 U/ml choline oxidase, 200 μM Amplex Red in 50 mM Tris, pH 8.0) was added to the plate using the BioRAPTR. The plate was centrifuged at 1000 rpm for 1 minute, incubated in the dark and read on a Perkin Elmer Envision (Ex 555 and Em 590). The data shown in this figure is from the 5-hour time point. The data was analysed using CBIS. The curves shown here are from 3 different experiments on 3 different days.

active compounds were then submitted for testing in a panel of OP nerve agents for confirmation. With confirmations from this panel testing and ADMET predictions using Biotechnology High Performance Computing Software Applications Institute (BHSI) software, chemical lead optimization from Compound 8 resulted in several novel oxime reactivator compounds with greater potency. One of these novel oxime reactivators, Compound 127, is more potent and faster acting than the current gold standard reactivator HI-6 in our assays.

Conclusion

Our primary goal for developing this high-throughput AChE reactivation assay was to discover novel reactivators of organophosphate poisoned acetylcholinesterase (AChE-OP) with improved broad spectrum against OP nerve agents vs 2-PAM and HI-6. The assay described in this paper allows quick analysis of test compounds in a convenient and safe 1536-well format. The assay can be used for either a high-throughput single concentration screen using 2 time points or for dose responses as described in this paper. Here, we validated the assay with 42 oxime reactivator test compounds selected from a virtual screen, along with 2 known reactivators, namely 2-PAM and HI-6. One of the compounds from the validation screen (Compound 8) had an $EC_{50} = 4.1 \mu M$ ($N=3$). Further analogue synthesis and structure activity optimization from Compound 8 led our team to Compound 127, which demonstrated rapid reactivation of AChE-OP in our NIMP assay with potency equivalent to HI-6. The translational relevance of the NIMP assay reactivator activity of Compound 127 from testing across a panel of OP nerve agents, including sarin, will be detailed in a subsequent report. Thus, this assay provides a critically needed tool to advance the identification of novel AChE reactivators.

Declaration of competing interest

The authors of this publication have no financial or personal interests that could potentially influence the work or create a conflict of interest.

Funding

This work was sponsored by the U.S. Government under Other Transaction number W15QKN-16-9-1002 between the MCDC and the Government. The US Government is authorised to reproduce and distribute reprints for Governmental purposes, notwithstanding any copyright notation thereon.

(Supplement Figures)

References

1. Klaassen C. Casarett and Doull's Toxicology: The Basic Science of Poisons. 9th ed. New York: McGraw-Hill; 2001;763–810. Available from: <https://accesspharmacy.mhmedical.com/book.aspx?bookid=2462>
2. Taylor P, Radic Z, Hosea NA, Camp S, Marchot P, Berman HA. Structural bases for the specificity of cholinesterase catalysis and inhibition. *Toxicol Lett.* 1995;82–83:453–8. Available from: [https://doi.org/10.1016/0378-4274\(95\)03575-3](https://doi.org/10.1016/0378-4274(95)03575-3)
3. Aldridge WN, Reiner E. Enzyme Inhibitors as Substrate Interactions of Esterases with Esters of Organophosphorus and Carbamic

Acids. Amsterdam: North-Holland Publishing Company; 1972.

Available from: <https://portlandpress.com/biochemsoctrans/article-abstract/2/3/567/58128/Enzyme-Inhibitors-as-Subst...>

4. Nagao M, Takatori T, Matsuda Y, Nakajima M, Iwase H, Iwadate K. Definitive evidence for the acute sarin poisoning diagnosis in the Tokyo subway. *Toxicol Appl Pharmacol.* 1997;144:198–203. Available from: <https://doi.org/10.1006/taap.1997.8110>
5. Kuhnen H. Activating and inhibitory effects of bispyridinium compounds on bovine red cell acetylcholinesterase. *Toxicol Appl Pharmacol.* 1971;20(1):97–104. Available from: [https://doi.org/10.1016/0041-008x\(71\)90093-7](https://doi.org/10.1016/0041-008x(71)90093-7)
6. Shih TM, Skovira JW, O'Donnell JC, McDonough JH. Evaluation of nine oximes on in vivo reactivation of blood, brain, and tissue cholinesterase activity inhibited by organophosphorus nerve agents at lethal doses. *Toxicol Mech Methods.* 2009;19:386–400. Available from: <https://doi.org/10.1080/15376510903213892>
7. Schopfer LM, Lockridge O. Analytical approaches for monitoring exposure to organophosphorus and carbamate agents through analysis of protein adducts. *Drug Test Anal.* 2012;4:246–61. Available from: <https://doi.org/10.1002/dta.1325>
8. Newmark J. Therapy for nerve agent poisoning. *Arch Neurol.* 2004;61:649–52. Available from: <https://doi.org/10.1001/archneur.61.5.649>
9. Wilson IB, Ginsburg B. A powerful reactivator of alkylphosphate-inhibited acetylcholinesterase. *Biochim Biophys Acta.* 1955;18:168–70. Available from: [https://doi.org/10.1016/0006-3002\(55\)90040-8](https://doi.org/10.1016/0006-3002(55)90040-8)
10. Mercey G, Verdet T, Saint-André G, Gillon E, Wagner A, Baati R, et al. First efficient uncharged reactivators for the dephosphorylation of poisoned human acetylcholinesterase. *Chem Commun (Camb).* 2011;47(18):5295–7. Available from: <https://doi.org/10.1039/c1cc10787a>
11. Mundy PM, Hamilton MG, Sawyer TW, Mikler J. Comparative protective effects of HI-6 and MMB-4 against organophosphorous nerve agent poisoning. *Toxicology.* 2011;285:90–6. Available from: <https://doi.org/10.1016/j.tox.2011.04.006>
12. Shih TM, Skovira JW, O'Donnell JC, McDonough JH. Evaluation of nine oximes on in vivo reactivation of blood, brain, and tissue cholinesterase activity inhibited by organophosphorus nerve agents at lethal dose. *Toxicol Mech Methods.* 2009;19:386–400. Available from: <https://doi.org/10.1080/15376510903213892>
13. Amitai G, Plotnikov A, Chapman S, Lazar S, Gez R, Loewenthal D, et al. Non-quaternary oximes detoxify nerve agents and reactivate nerve agent-inhibited human butyrylcholinesterase. *Commun Biol.* 2021 May 14;4(1):573. Available from: <https://doi.org/10.1038/s42003-021-02061-w>
14. Ohta H, Ohmori T, Suzuki S, Ikegaya H, Sakurada K, Takatori T. New safe method for preparation of sarin-exposed human erythrocytes acetylcholinesterase using non-toxic and stable sarin analogue isopropyl p-nitrophenyl methylphosphonate and its application to evaluation of nerve agent antidotes. *Pharm Res.* 2006;23:2827–33. Available from: <https://doi.org/10.1007/s11095-006-9123-1>
15. Meek EC, Chambers HW, Coban A, Funck KE, Pringle RB, Ross MK, et al. Synthesis and in vitro and in vivo inhibition potencies of highly relevant nerve agent surrogates. *Toxicol Sci.* 2012;126:525–33. Available from: <https://doi.org/10.1093/toxsci/kfs013>
16. Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal Biochem.* 1997;253:162. Available from: <https://doi.org/10.1006/abio.1997.2391>
17. Mohanty JG, Jaffe JS, Schulman ES, Raible DG. A highly sensitive fluorescent micro-assay of H2O2 release from activated human leukocytes

using a dihydroxyphenoxazine derivative. J Immunol Methods. 1997 Mar 28;202(2):133–41. Available from: [https://doi.org/10.1016/s0022-1759\(96\)00244-x](https://doi.org/10.1016/s0022-1759(96)00244-x)

18. Benedict RC, Stedman RL. Ellman's reagent: interference in mercapto group determination, with special reference to cigarette smoke. Analyst. 1970;95(1128):296–301. Available from: <https://pubs.rsc.org/en/content/articlelanding/1970/an/an9709500296>

19. Zhang JH, Chung TD, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen. 1999;4:67–73. Available from: <https://doi.org/10.1177/108705719900400206>

Discover a bigger Impact and Visibility of your article publication with Peertechz Publications

Highlights

- ❖ Signatory publisher of ORCID
- ❖ Signatory Publisher of DORA (San Francisco Declaration on Research Assessment)
- ❖ Articles archived in worlds' renowned service providers such as Portico, CNKI, AGRIS, TDNet, Base (Bielefeld University Library), CrossRef, Scilit, J-Gate etc.
- ❖ Journals indexed in ICMJE, SHERPA/ROMEO, Google Scholar etc.
- ❖ OAI-PMH (Open Archives Initiative Protocol for Metadata Harvesting)
- ❖ Dedicated Editorial Board for every journal
- ❖ Accurate and rapid peer-review process
- ❖ Increased citations of published articles through promotions
- ❖ Reduced timeline for article publication

Submit your articles and experience a new surge in publication services

<https://www.peertechzpublications.org/submission>

Peertechz journals wishes everlasting success in your every endeavours.