UMURJ
University of Michigan Undergraduate Research Journal

Oxime-Conjugated Dendrimers as Therapeutics for Organophosphate Poisoning

RACQUEL HARRISON[a], TANG SHENGZHUANG[ab], JAYME CANNON [ab], KELLY YANG[a], PAMELA WONG[ab], SEOK KI CHOI[ab]

Organophosphates (OPs) are a class of organophosphorus compounds which have a general structure of $O=P(OR)_3$. Paraoxon (also known as POX) is an organophosphate found in insecticides often used in agriculture in impoverished countries. OPs are also used in nerve gas agents such as Sarin gas, used in warfare.These molecules are extremely toxic and can be lethal at low doses. Organophosphates negatively impact the body's neurotransmitter enzyme, acetylcholinesterase. Muscle spasms, paralysis, narrowed pupils, glossy eyes, confusion, and the over-secretion of bodily fluids such as sweat, saliva, mucus, and tears, are symptoms of organophosphate poisoning (Fletcher, 2017). To reduce the harmful effects from this organic compound, we have designed and screened dendrimer nanoparticles modified with poly (ethylene glycol) chains and with small reactor molecules. Dendrimers are synthetic macromolecules, which contain chains of repeated, branching molecules that form a tree-like or snowflake-like structure. Dendrimers can be used to enhance permeability in drug delivery. There are many active sites attached to small reactor molecules on the dendrimer branches, allowing for the successful and efficient inactivation of POX before penetrating the skin (Mukherjee, 2015). The second focus of our experiment was to screen for hundreds of oxime molecules that most effectively reactivate acetylcholinesterase. Oxime molecules are an organic imine compound that can be used to detach the toxic OPs from acetylcholinesterase. OP's bind to Ser^{200} on the enzyme, leading to inactivation. Oxime molecules bind in a nucleophilic attack to the phosphorus on organophosphate, causing it to diffuse away from the enzyme. This paper highlights the two focuses of our experiment: 1. Perform enzyme assays to screen for the best compounds at reactivating acetylcholinesterase, and 2. Attach oxime molecules at the branching sites of dendrimers to enhance permeability into

Contact: Racquel Harrison, Tang Shengzhuang, Jayme Cannon, Kelly Yang, Pamela Wong, Seok Ki Choi <racquelh@umich.edu>

https://doi.org/10.3998/umurj.3782

the subdermal layer of the skin. We have identified some potential oxime molecules and dendrimers to be complexed and used as a treatment for OP poisoning or incorporated into a cream-based drug product to protect those at high risk of exposure to organophosphates.

Introduction

Acetylcholine (ACh) is a small signaling molecule released in a synapse from the axon of nervous cells. ACh binding to its receptor sends chemical impulses to other nerve cells. Acetylcholinesterase (AChE) breaks down an excess of acetylcholine after a sufficient stimulation on the neighboring nerve cell is made. If the enzyme is inhibited by organophosphates (OPs), the remaining ACh continues to stimulate the nerves, resulting in diarrhea, hypertension, muscle spasms, etc. (Fletcher, 2017). The neuropsychiatric effects of organophosphate poisoning include another extensive list of symptoms including impaired memory, confusion, irritability, lethargy, psychosis, etc. There are almost 3 million poisonings per year with a result of 200,000 deaths (Eddleston, 2002, p. 275).

The phosphorus atom in the organophosphate binds to the hydroxyl group in the active site of acetylcholinesterase, deactivating the enzyme. Oxime molecules, with a general formula RR'C=NOH, also contain a hydroxyl group (J. Biol. Chem, 2011, p. 286). When in the presence of the poisonous compound, the hydroxyl group of the oxime molecule does a nucleophilic attack on OP's phosphorous, causing it to detach from the enzyme (Eddleston, 2002, p. 275). This oxime-phosphonate formation is an irreversible reaction, allowing AChE to remain activated. Below are some examples of reference oxime compounds already used commercially.

Although effective, the side effects of oximes include vomiting, nausea, confusion, dry eyes and mouth and muscle weakness (Institute of Medicine, 2000). Our goal is to reduce the harmful effects of these OPs by designing a topical cream that can be used in the event of dermal exposure to organophosphates, particularly

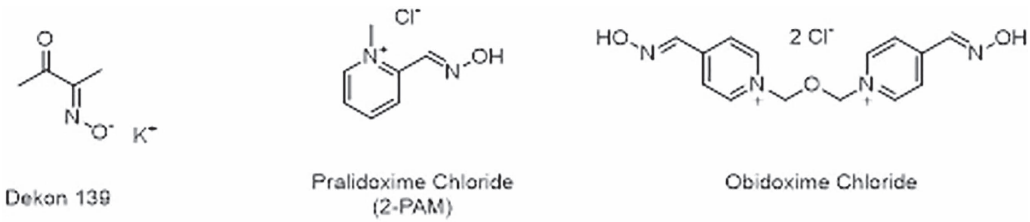

Figure 1: Three different oxime molecules that have been patented and used commercially against organophosphate toxicity.

in the agricultural sector. We hypothesize that the use of oxime-conjugated dendrimers will reverse the effects of the OPs, allowing for the reactivation of acetylcholinesterase. By screening different oxime small molecules, we can compare the enzymatic reactivity of AChE with other post-market products such as 2-PAM and Obidoxime. Within our experiments, we tested the reactivity of acetylcholinesterase after exposure to POX, and we designed a system to test skin penetration of the dendrimers using Franz cells and Ultra-Pressure Liquid Chromatography.

Methods

AChE Enzyme Reactivation Assays

Assays are used to test how effectively the oximes reactivate the enzyme acetylcholinesterase after it has been deactivated by paraoxon. The frozen enzyme is first thawed. The AChE is then vortexed with one-part enzyme, 499 parts pH 8 PBS. The amount of enzyme needed varies depending on how many oximes will be tested. We diluted POX with a serial dilution to test it at different concentrations. A serial dilution like this can be used to create a calibration curve of POX concentration vs enzymatic reactivity. In pH 8 PBS, we dilute the organophosphate anywhere from 20 μM to 0.156 μM. In the following order, solutions are added to the 96 well plates: pH 8 PBS (50, 70 or 80 μL), AChE (100 μL), and POX (10 μL). The plates are then incubated for 30 minutes to let the POX deactivate the enzyme AChE. Afterwards, 20 μL of the different oxime conjugates are added to each row (except for the control row), ranging anywhere from 0.01mM to 10mM. Our oxime molecules were synthesized in the lab apart from 2-PAM and Obidoxime chloride, which were purchased through Fisher Scientific. Although the synthesis of attaching the oxime molecules to the PAMAM dendrimers is not discussed here, Bharathi's (2014) research and design on oxime-conjugated PAMAM dendrimers was modeled. (Bharathi, p. 1068–1078). We used Obidoxime and 2-PAM as controls, which are already marketed oxime products. A fresh 1:1 mixture of acetylthiocholine chloride, ATCh, and 5,5'-dithio-bis (2-nitrobenzoic acid), DTB, are weighed out and dissolved into DMSO and PBS 8.0. This 1:1 mixture of ATCh (5.0 mM, 3.0 mL) and DTNB (5 mM, 3.0 mL) is labeled as "AT/DT," and it shall be used as a substrate to measure the enzymatic reactivity of acetylcholinesterase. 20 μL of the substrate is pipetted swiftly into each well with a multi-channel pipette. Prior to adding the substrate, the plate reader is set to an absorbance of 412nm and to read enzymatic activity every 2 minutes for 20 minutes. Enzymatic activity is measured by detecting absorbance via color change from clear to yellow upon addition of the AT/DT mixture. The higher the reactivity of the enzyme

acetylcholinesterase, the darker the yellow would appear in the wells after the reaction has reached completion. Figure 2 shows an example of a 96 well plate setup that was tested:

Plate 706–41C series (each well 200 µL)

- Buffer, PBS pH 8.0: variable volume (80, 70 or 50 µL)
- AChE: 100 µL each fixed
- POX = 10 µL each fixed volume
  - **Row A: variable concentration**
  - **Row B to H: 10 µM**
- Test oxime solution: 20 µL each, variable concentration (0.01–10 mM) as noted in the upper row
- AT/DT: 20 µL each fixed

POX decontamination in porcine skin tissue

We test how long it takes for the POX to be removed from the skin using Franz cells, which clamp the skin tissue in place. Pig skin is used for this experiment because it is most similar to human skin. In this experiment, we recreated a makeshift human-synapsis with a pH buffer of 7.4 absorbing the internal side of our pig skin, since the human body has a natural pH of around 7.4. We add

706-41C	1	2	3	4	5	6	7	8	9	10	11	12
	Oxime= 0.25mM			Oxime= 0.5mM			Oxime= 2.0mM			Oxime= 5.0mM		
A. Control	B(80)+AChE(100)+No POX			B(70)+AChE(100)+POX(0.1µM)			B(70)+AChE(100)+POX(1.0µM)			B(70)+AChE(100)+POX(10µM)		
B. 2-PAM	B(50)+AChE(100)+POX(10)+ 2PAM(20)			B(50)+AChE(100)+POX(10)+ 2PAM(20)			B(50)+AChE(100)+POX(10)+ 2PAM(20)			B(50)+AChE(100)+POX(10)+ 2PAM(20)		
C. Obidoxi	B(50)+AChE(100)+POX(10)+ Obidoxime(20)			B(50)+AChE(100)+POX(10)+ Obidoxime(20)			B(50)+AChE(100)+POX(10)+ Obidoxime(20)			B(50)+AChE(100)+POX(10)+ Obidoxime(20)		
D. 693-3a	B(50)+AChE(100)+POX(10)+ 693-3a(20)			B(50)+AChE(100)+POX(10)+ 693-3a(20)			B(50)+AChE(100)+POX(10)+ 693-3a(20)			B(50)+AChE(100)+POX(10)+ 693-3a(20)		
E. 693-3b	B(50)+AChE(100)+POX(10)+ 693-3b(20)			B(50)+AChE(100)+POX(10)+ 693-3b(20)			B(50)+AChE(100)+POX(10)+ 693-3b(20)			B(50)+AChE(100)+POX(10)+ 693-3b(20)		
F. 693-8a	B(50)+AChE(100)+POX(10)+ 693-8a(20)			B(50)+AChE(100)+POX(10)+ 693-8a(20)			B(50)+AChE(100)+POX(10)+ 693-8a(20)			B(50)+AChE(100)+POX(10)+ 693-8a(20)		
G. 693-8b	B(50)+AChE(100)+POX(10)+ 693-8b(20)			B(50)+AChE(100)+POX(10)+ 693-8b(20)			B(50)+AChE(100)+POX(10)+ 693-8b(20)			B(50)+AChE(100)+POX(10)+ 693-8b(20)		
H. 693-19a	B(50)+AChE(100)+POX(10)+ 693-19a(20)			B(50)+AChE(100)+POX(10)+ 693-19a(20)			B(50)+AChE(100)+POX(10)+ 693-19a(20)			B(50)+AChE(100)+POX(10)+ 693-19a(20)		

Figure 2: A typical well plate set up to screen for oxime conjugates. Some of the oxime small molecules that we narrowed our focus on were 693–3a, 693–3b, 693–8a, 693–8b, 693–19a, 693–19b, 693–70a, 693–70b, 693–29a, 693–29b, 693–29c, 693–29d, 693–29d, 693–77a, 693–77b, 693–77c, 693–77d, 693–77e, and 693–77f.

a buffer of pH 10.5 to the donor side, the exterior side of the skin, allowing it to equilibrate. We then remove the pH 10.5 buffer and add the paraoxon (a type of organophosphate) to the outer surface of the skin. We incubate the OP on the skin for 2 hours to allow for full deactivation of AChE. After the incubation, we add 300 microliters of the dendrimers to the top of the Franz cell. Once the dendrimers are added, we remove 50 microliter samples at indicated time points and add them to formic acid to quench the reaction. The usual time points used are at t=0 minutes, 6 minutes, 15, 30, 60, and 120 minutes. Ultra-Pressure Liquid Chromatography (UPLC) is used to separate small compounds into complex mixtures. Using UPLC analysis, we want to see the levels of activated POX decreasing over time. We expect almost 100% of POX at 0 minutes and deactivation of nearly all POX at 120 minutes. One way we measure this is by doing AUC, or area under the curve. The smaller the peak's area that shows up on the UPLC, the less POX remains. We also took samples of the pH 7.4 buffer on the receptor side of the skin to see how much of the POX went through the skin. This is also measured by UPLC. Next, a skin extraction is performed: we soak the decontaminated skin in ethanol overnight. The following day, a sample of the ethanol was removed and tested by UPLC to see how much POX and dendrimer remained in the epidermal and basal layers of the skin.

Results

Enzyme reactivation

We found that the higher the enzyme percent reactivity, the more effective the oxime is at cleaving the organophosphate from the active site. We expect to see higher reactivity at lower concentrations for the use of potential small molecule drug products.

In the graphs, we strive to see high enzyme reactivity, meaning that the dendrimer compound would effectively and efficiently deactivate the POX and reactive the enzyme. We see high enzyme reactivation in compounds 693–29f, 693–3b, 693–8b, 693–77c, and 693–77b (Figure 3). Not only were these molecules most effective but they showed the most consistency when the data was repeated. After addition of substrate, our pre-set Bioassay Plate Reader takes 25 absorbance readings in a span of 20 minutes. The kinetic rate of the enzymatic reaction is interpreted on a logarithmic scale. In Microsoft Excel, the initial slope is calculated for each oxime compound at various concentrations. Three data points are taken at each indicated oxime concentration; the average and standard deviation of the three absorbance values are calculated. Each 96-well setup contains a control row where no oxime is added (Figure 2). The first three columns

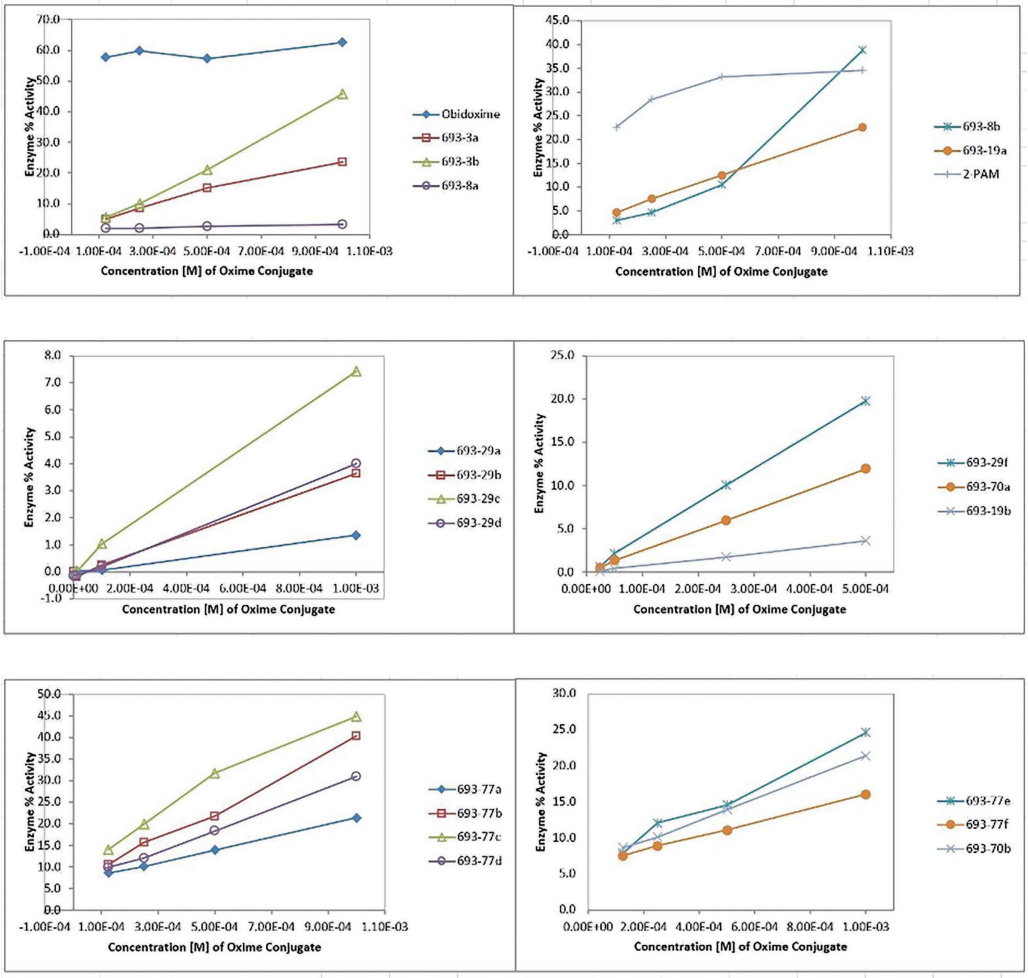

Figure 3: Results of 21 different oxime conjugates, two of which (Obidoxime and 2-PAM) are marketed products of OP poisoning. The data in these graphs come from plate assays using 96 well plates. On the x-axis, we have the concentration [M] of the oxime being added to the wells. On the y-axis, we have the percent reactivation of the enzyme calculated by the equation 100*[k-k(inactive)]/[k(active)-k(inactive)]. 'k' is the mean reactivity of the enzyme or each conjugate, 'inactive' is a constant for when the enzyme is least reactive, and 'active' is a constant for when the enzyme is most active. Enzyme % activity is relative to the control.

of the control row contain no POX (wells A1, A2 and A3), and the last three columns contain the highest concentration of POX (wells A10, A11, and A12). Two averages of two slopes are found using these absorbance values: one is labeled "active" and the other "inactive." Setting k=slope, percent enzyme (re)activity is calculated using the equation: Enzyme % Activity=100*[k-k(inactive)]/[k(active)-k(inactive)]. The averages and variance values are then calculated for

each small oxime molecule at indicated concentrations. These averages are then plotted with concentration of the oxime conjugate on the x-axis and enzyme percent activity on the y-axis (Figure 3).

POX decontamination in porcine skin

The graphs shown below are results of UPLC (ultra-performance liquid chromatography) analysis for POX decontamination in the donor compartment of the Franz cell. In the skin decontamination experiment, our goal is to find a conjugate that will allow the least POX penetration through human skin.

Below is data collected from a UPLC of how long it took for POX to be fully inactivated on the surface of the porcine skin (Figure 4). The faster the dendrimer latches onto and deactivates the POX, the less likely the POX will penetrate through the skin to get into the bloodstream, meaning a lower toxicity level. The absorption unit (proportional to the POX remaining) was measured at 215nm and 275nm. The dendrimers eluted from the UPLC at a retention time of about 10.25 minutes, represented by the x-axis in Figure 4. An internal standard was used to calibrate the absorption unit of each dendrimer at the various time points (Absorption Unit is represented by the y-axis). The Area Under the Curve (AUC) was measured for each sample of each dendrimer pipetted from the Franz cells at various time points ranging from 0 to 120 minutes. Data taken by the UPLC are then transferred to an Excel spreadsheet where the results can be better displayed. As seen in Figure 4, the readings taken at 275 nanometers appear to show more sensitive results than those taken at 215 nanometers. The peaks on the graphs represent the remaining volume of POX on the apical side of the Franz cell. By adding the dendrimers, it is expected that the active form of the organophosphate will dissipate within 2 hours. The more ideal results would show a faster dissipation of the active form of POX. One example of an ideal candidate can be seen in the results 693–40A because there is no remaining POX after 1 hour of incubation with the dendrimer, whereas 706–8A is an example of a bad candidate because there continues to be traces of POX after 2 hours of incubation (Figure 4).

Discussion

As shown in Figure 3, the enzyme percent reactivity is consistently high at all concentrations of the 2-PAM and obidoxime. Our goal is to screen for an oxime molecule that shows high acetylcholinesterase reactivity especially at lower concentrations: if found, such an oxime would show high potency if used as the active pharmaceutical ingredient in a drug product. After about a year of

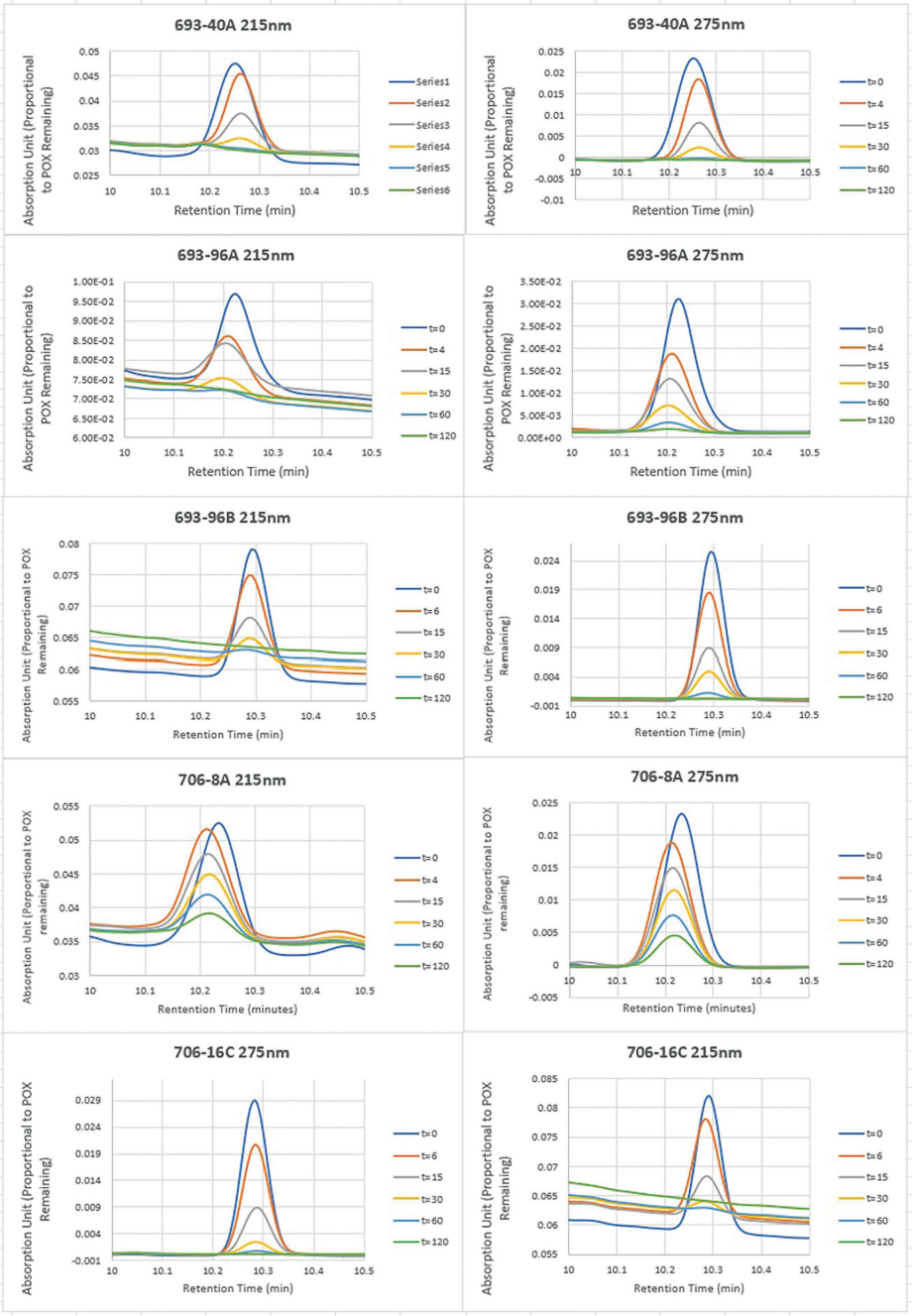

Figure 4: Ultra-Pressure Liquid Chromatography data taken at 215nm and 275nm for five different dendrimers. Time (t) is measured in minutes. Wavelengths 215 and 275 nanometers were used for the dendrimers because these wavelengths showed the best sensitivity of our analytes. 275 nanometers tended to show clearer "peaks" for most of our dendrimers compared to at 215 nanometers.

screening of the most effective oxime conjugates, we have decided to continue our investigations with 693–3b, 693–70a, 693–70b, 693–77a, 693–77b, 693–77c, 693–77d, 693–77e, and 693–77f. In the drug development process, thousands of compounds may be screened, but only a dozen or so make it to the preclinical testing phase. Moving forward, we will be using these oxime molecules in our preclinical studies. As hypothesized, we predicted that we would find promising oxime compounds via high throughput screening using enzyme assays.

As described in figure 4, the dendrimers 706–40a, 706–16c, and 706–96a most efficiently inactivated POX from the porcine skin during our Franz cell procedure. When analyzing the graphs, we notice in 706–8A that a large fraction of the POX remains in the porcine skin after 120 minutes (about 2 hours). Whereas 706–40A shows the complete removal of organophosphates from the skin after less than 60 minutes. If a person is poisoned by organophosphates, we want to make a topical substance that can be applied to the skin to destroy the POX, but not get into the bloodstream. Dendrimers are great for drug delivery, as they help enhance the permeability of the oxime conjugates by attaching them to the ends of the polymeric dendritic branches. Since there are many branches on these dendrimers, they can hold more oxime molecules per dendrimer, allowing for better delivery to the site of action. In the future, we hope to see comparable results when performing *ex vivo* and *in vivo* studies. Our approach is to make a topical cream to be used in the agricultural sector for dermal exposure to OP compounds. Although we do not plan to develop a product for organophosphate exposure via inhalation, we hope our findings can help advance the discovery of antidotes for inhalation exposure of OPs, including Sarin gas. We also strive to better understand the mechanistic action of our dendrimers, 706–16C, 706–40A, and 706–96A especially, our most effective compounds. We are beginning to transition from high throughput screening to preclinical studies using our results from our previous discoveries.

References

Baker J.R. Jr, Bharathi S., Choe, Choi S. K. V., Desai A., Kim H., Lykhytska O., Thomas T. P., Wong P. T., (2014). Design and mechanistic investigation of oxime conjugated PAMAM dendrimers as the catalytic scavenger of reactive organophosphate. *J Mater Chem B, 8*, 1068–1078.

Baker J.R. Jr, Choi S.K., Coulter A, Gam K, Mukherjee J, Tang S, Wong P.T. (2015). Mechanism of Cooperativity and Nonlinear Release Kinetics in Multivalent Dendrimer-Atropine Complexes. *Mol. Pharmaceutics, 12*, 4498–4508.

Eddleston, M et al. (2002). Oximes in acute organophosphorus pesticide poisoning: a systematic review of clinical trials. *QJM: monthly journal of the Association of Physicians, 95*, 5, 275–83.

Eddleston, Michael et al. (2008). Management of acute organophosphorus pesticide poisoning. *Lancet, 371*(9612), 597–607.

Fletcher, Jenna. (2017). What are the symptoms of organophosphate poisoning. *Medical News Today*.

Fokin V., Gerardi V., Radic Z., Sharpless K. Barry., Sit Rakesh K., Taylor P. (2011). New Structural Scaffolds for Centrally Acting Oxime Reactivators of Phosphylated Cholinesterases. *J. Biol. Chem, 286* (22), 19422–19430.

Fulco C.E., Liverman C.T., Sox H.C. (2000). Effects of Long-Term Exposure to Organophosphate Pesticides in Humans. Institute of Medicine (US) Committee on Health Effects Associated with Exposures During the Gulf War; *Gulf War and Health, 1*.

Fokin V., Radic Z., Sharpless K. Barry., Sit Rakesh K., Taylor P., Zrinka K. (2012). Refinement of Structural Leads for Centrally Acting Oxime Reactivators of Phosphylated Cholinesterases. *J. Biol. Chem., 287*, 11798–11809.

U.S. Department of Health and Human Services (2019). Obidoxime- Medical Countermeasures Database. Chemical Hazards Emergency Medical Management. *Chemical Hazards Emergency Medical Management*.