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# Alternative therapeutic mechanisms of novel phenoxyalkyl pyridinium oximes to treat organophosphorus compounds

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# Alternative therapeutic mechanisms of novel phenoxyalkyl pyridinium oximes to treat organophosphorus compounds

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Alternative therapeutic mechanisms of novel phenoxyalkyl pyridinium oximes to treat organophosphorus compounds

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Organophosphates (OPs), such as nerve agents and insecticides, potently inhibit acetylcholinesterase (AChE). Oximes, such as the currently FDA approved oxime 2-PAM, remove the OP from the inhibited enzyme. 2-PAM is effective against select OPs and cannot effectively pass the blood-brain barrier to attenuate OP induced CNS damage. Our laboratory has synthesized a series of substituted phenoxyalkyl pyridinium oximes (Patent number: 9,227,937) that have demonstrated increased survival rates compared to 2-PAM. This research investigated 1) *in vitro* oxime reactivation of rat, human, and guinea pig serum BChE after inhibition by nerve agent and insecticidal OPs; 2) *in vitro* determination of reactivation kinetic rate constants for OP inhibited human and rat serum BChE and electric eel AChE after inhibition by a sarin surrogate and paraoxon; 3) intranasal delivery of oximes to reactivate brain AChE *in vivo* after inhibition by a sarin surrogate. Novel oxime 15 demonstrated significant broad spectrum reactivation of OP-inhibited rat serum BChE while novel oxime 20 demonstrated significant broad spectrum reactivation of OP-inhibited human serum BChE. All tested oximes were poor reactivators of OP-inhibited guinea pig serum BChE. Kinetic analysis of reactivation for NIMP and paraoxon human and rat serum BChE and electric eel AChE demonstrated differences in the

second order rate constants. Oxime 20 demonstrated reactivation efficiency for both NIMP and paraoxon inhibited rat and human serum BChE and electric eel AChE more effectively than 2-PAM. Intranasal delivery of either oxime 20 or 2-PAM showed attenuation of NIMP-inhibited brain AChE inhibition in select brain regions and select time points. Oxime 20 demonstrated a larger window of effectiveness but neither oxime attenuated brain AChE inhibition in the hindbrain for any time point or for any brain region at the ten minute time point. These data suggest that reactivation of OP-inhibited BChE may be contributing to the observed increases in survival seen with our oximes. Novel oxime 20 demonstrated reactivation efficacy towards both BChE and AChE inhibited enzyme and a rapid entry into the brain after intranasal delivery. Having an oxime that can be effective in a multitude of ways would be of great value to medical and military personnel.

DEDICATION

This work is dedicated to my family, especially my mom and dad. Thank you for always being there for me. You have never stopped supporting me, even when I was struggling. I greatly appreciate all that you have done for me. You are the best parents a kid could ask for! I love you very much.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

| | |
|---|---|
| 2-PAM | *2-pyridinium aldoxime* |
| ACh | *Acetylcholine* |
| ATCh | *Acetylthiocholine* |
| AChE | *Acetylcholinesterase* |
| BBB | *Blood brain barrier* |
| BTCh | *Butyrylthiocholine* |
| BChE | *Butyrylcholinesterase* |
| CNS | *Central nervous system* |
| DMSO | *Dimethyl sulfoxide* |
| DTNB | *5,5'-dithio bis(2-nitrobenzoic acid)* |
| DFP | *Diisopropyl fluorophosphate* |
| EtOH | *Ethanol* |
| FC | *Final concentration* |
| GABA | *Gamma amino butyric acid* |
| HI-6 | *Asoxime* |

| | |
|---|---|
| $LD_{50}$ | *Lethal dose at which 50% of the animals expire* |
| MINA | *Monoisonitrosoacetone* |
| MMB-4 | *Methoxime* |
| NCMP | *Nitrophenyl cyclohexyl methylphosphonate* |
| NEMP | *Nitrophenyl ethyl methylphosphonate* |
| NIMP | *Nitrophenyl isopropyl methylphosphonate* |
| NMR | *Nuclear magnetic resonance* |
| OBD | *Obidoxime* |
| OPs | *Organophosphorus compounds* |
| PIMP | *Phthalimidyl isopropyl methylphosphonate* |
| PHO | *Phorate oxon* |
| PHOsox | *Phorate oxon sulfoxide* |
| PNS | *Peripheral nervous system* |
| PXN | *Paraoxon* |
| SDS | *Sodium dodecyl sulfate* |
| SLUD | *Salivation, lacrimation, urination, defecation* |
| TEPP | *Tetraethyl pyrophosphate* |
| TMB-4 | *Trimedoxime* |

CHAPTER I

INTRODUCTION

## Organophosphate History and Usage

Organophosphorus compounds (OPs) are a diverse group of organic chemicals which share a central phosphorus (P) atom and 3 other atoms with usually one double bond and two single bonds. The atoms vary significantly surrounding the P atom but are most typically oxygens (O) and sulfurs (S). The first OP to be synthesized was triethyl pyrophosphate in 1820 by Lassaigne followed shortly by tetraethyl pyrophosphate (TEPP) by de Clermont in 1854 (Chambers *et al.*, 1992). It would not be until almost 100 years later when the insecticidal properties of TEPP were revealed by Gerhard Schrader, a German scientist who was investigating new insecticides. Schrader and others went on to develop some very toxic OP compounds, namely tabun, sarin, soman, and parathion (Chambers *et al.*, 1992). Tabun, sarin, and soman showed high acute mammalian toxicity and volatility and were deemed too toxic to be developed as insecticides but instead Schrader was instructed by the Nazi government to develop them as chemical weapons to be used in warfare. These chemical weapons were called nerve agents, as they attack the nervous system. Various nerve agents have been synthesized and are categorized as 'G' agents or 'V' agents. The 'G' agents, which the 'G' stands for German, consist of sarin (GB), tabun (GA), soman (GD), and cyclosarin (GF). The 'V' agents, namely VX, which the 'V' stands for venomous, were originally synthesized by Britain and the United States in the 1950s. Russian and Chinese derivatives of VX, VR and CVX, respectively, were later synthesized (Antonijevic and Stojilijkovic, 2007). The G agents differ from the V agents

based on their chemical reactivity and toxicity. The G agents are clear liquids when fresh that have high volatility whereas the V agents are viscous, have low volatility, are more environmentally persistent, and are typically amber colored (Busl & Bleck, 2012). The V agents are generally more toxic (rat iv $LD_{50}$ 7-10µg/kg for VX) than the G agents (rat iv $LD_{50}$ 45-63µg/kg for sarin; John *et al.*, 2009). Exposure primarily occurs from either inhalation or dermal exposure.

Parathion was developed as an insecticide to counteract the growing insect resistance to the then used organochlorine compounds. Various other OP pesticides were synthesized throughout this timeframe and the OP class of compounds became the leading class of insecticides for several decades until their replacement by the less toxic synthetic pyrethroids (Chambers *et al.*, 1992). OP pesticides in general are less toxic than the nerve agents; however, there are a few insecticidal OPs that are quite toxic, such as parathion and phorate. One of the differences between OP pesticides and nerve agents is that several OP pesticides, including parathion and phorate, require metabolic activation from the double bonded sulfur attached to the P atom (P=S) to the 'oxon' (paraoxon and phorate oxon, respectively) which replaces the sulfur with an oxygen (P=O) and increases the toxicity of the compound (Hayes and Laws, 1991; Chambers *et al.*, 1992; Moyer *et al.*, 2018). This reaction, called oxidative desulfuration, is carried out by cytochrome P450 enzymes, a superfamily of phase 1 metabolizing enzymes found throughout the entire body. The nerve agents already possess the P=O functionality and therefore do not require any activation to be toxic.

Because of the high mammalian toxicity, relative ease of synthesis, and difficulty of treatment associated with nerve agents and OP pesticides, they pose a risk for warfighters and

civilians. They are an attractive choice for rogue dictators and terrorist groups and, despite being banned for use in warfare, have been used in several instances. During the Iran-Iraq war, radical dictator Saddam Hussein authorized the release of nerve and mustard agents on Iranian and Kurdish populations, killing and injuring several thousand (Rose and Baravi, 1988; Balali-Mood and Balali Mood, 2008; Smart, 1997). In Tokyo, 1995, the terrorist group Aum Shinrikyo released sarin in the subway system killing several and wounding several thousand (Nagao *et al.,* 1997; Yokoyama *et al.,*1998). More recently, in 2013, nerve agents were used in the Syrian conflict which resulted in the deaths of thousands of people (Dolgin, 2013; Pita and Domingo, 2014).

While the threat to life from exposure to OP nerve agents is significant, the likelihood of exposure to such compounds is minimal. On the other hand, OP pesticides still present a problem to clinicians in the United States and worldwide every year. OP insecticides are an effective and inexpensive weapon against insects of all types and, despite decreased usage in the United States, still represent 33% of the US pesticide market, which suggests exposure to OPs is still possible (Atwood and Paisley-Jones, 2017). In underdeveloped countries, where training in the safe handling and transport of pesticides is poor, and education on the harmful effects of pesticides is limited, or where less stringent regulations on sale and storage of pesticides are present, or where illegal sales of pesticides have occurred, accidental exposures and subsequent deaths of children and agriculture workers happen fairly frequently (Chowdhury *et al.,* 2008; Rosenthal, 2003; Leverton *et al.,* 2007; Tsai *et al.,* 2007). Unfortunately, the more common scenario of pesticide poisoning in developing countries is self ingestion to commit suicide, many of them being due to OPs (Bertolote *et al.,* 2006; Chugh *et al.,* 1991; Eddleston, 2000; Fitzgerald *et al.,* 1978). It is

estimated that 200,000 people die every year to OP poisoning, mostly in underdeveloped countries (Eddleston and Chowdhury, 2016).

## Mechanism of Action

The toxicity of OPs can be largely attributed to the inhibition of acetylcholinesterase (AChE; EC 3.1.1.7), a serine hydrolase responsible for hydrolyzing the neurotransmitter acetylcholine (ACh), a chemical messenger that mediates several critical functions in both the peripheral and central nervous systems. AChE is an exceptionally fast catalyst, operating at the speed limit of biological catalysts, typically hydrolyzing ACh molecules with a $k_{cat} > 10^4 s^{-1}$ in a two-step reaction that takes place in less than a millisecond (Quinn *et al.*, 1987; Quinn *et al.*, 2017; Rosenberry, 1975). The first part of the reaction is a transesterification step, also called the acetylation step, where the ester bond in the acetylcholine is attacked by the nucleophilic amino acid serine in acetylcholinesterase, allowing the choline to depart and be recycled and the formation of an acetyl-enzyme complex. The next step is a hydrolysis step, also called deacetylation, where a water molecule is activated by the amino acid histidine, which attacks the acetyl-enzyme complex and hydrolyzes the ester linkage. This reaction forms an acetic acid and free enzyme (Mercey *et al.*, 2012). OP inhibition mimics the acetylation step. The nucleophilic serine attacks the phosphorus atom, allowing the leaving group to depart and forming a phosphyl-serine complex. Hydrolysis of the OP is slow (hours to days) due to steric hindrances of water molecule activation (Mercey *et al.*, 2012); therefore, the enzyme is persistently inhibited (see Figure 1-1 for ACh hydrolysis and OP inhibition of AChE).

## Clinical Effects

The persistent OP induced inhibition of AChE leads to a buildup of Ach at neuronal synapses and neuromuscular junctions. The actions of Ach on nicotinic and muscarinic cholinergic receptors are amplified leading to a systemic cascade of toxic responses called the cholinergic toxidrome. The clinical signs are diverse because of cholinergic innervation in the parasympathetic and sympathetic nervous systems. Excessive stimulation of nicotinic receptors at neuromuscular junction sites in skeletal muscle tissue causes muscle spasms, twitching, and eventual paralysis of important respiratory muscles such as the intercostal muscles and the diaphragm, while activation of muscarinic receptors leads to increased gland secretions at salivary glands, mucosal glands, eye glands, depressed heart rate, bronchial airway constrictions, and increased GI tract movement (Kellar, 2006). Clinical signs include shortness of breath, hypo- and hypertension, tremors, seizures, muscle weakness, tachycardia and bradycardia, abdominal cramps, miosis, salivation, lacrimation, urination, defecation (SLUD), laryngospasm, bronchorrhea, rhinorrhea, and, ultimately, death occurs from respiratory failure (Antonijevic and Stojilijkovic, 2007).

## Clinical Treatment

There are three strategies to treat organophosphate poisoning. They consist of anticholinergics, oximes, and anticonvulsants. The anticholinergic used is atropine sulfate. Atropine is a competitive antagonist of muscarinic receptor subtypes which attenuates the bradycardia and excessive gland secretions in the peripheral nervous system. However, atropine is limited in its effectiveness because of its inability to reduce nicotinic receptor stimulation, its limited effectiveness at attenuating muscarinic stimulation in the CNS, and the fact that high

amounts of Ach in the case of high dose OP exposure can outcompete the atropine on muscarinic receptors (Antonijevic and Stojilijkovic, 2007).

Anticonvulsants, such as diazepam or midazolam, are used to attenuate seizures. These drugs belong to a class called benzodiazepines which are agonists of GABA receptors, the primary inhibitory neurotransmitter in the brain. This increase of inhibitory drive causes a hyperpolarization of neurons, making them less susceptible to cholinergic and glutamatergic stimulation (Shih, 1991; McDonough *et al.,* 2000). Oximes are used to remove the OP from the enzyme in a process called reactivation because AChE becomes functional again and can attenuate the excessive accumulation of Ach, allowing the body to return to homeostasis (Kusic *et al.,* 1991; Thiermann *et al.,* 1997; Thiermann *et al.,* 1999). There exists a variety of oxime platforms. The currently approved oxime treatment in the United States is pyridinium-2-aldoxime, or 2-PAM. Other pyridinium based oximes, such as the bis-pyridinium oximes TMB-4, MMB-4, obidoxime (OBD), HI-6, and others, are used in other nations (see Figure 1-2 for oxime structures). What makes oximes work is the formation of a nucleophilic oximate anion (Mercey *et al.,* 2012). These nucleophilic functional groups remove the electrophilic phosphoryl group of the OP from the catalytic serine in AChE (see Figure 2 for oxime mechanism), which causes the OP to form a stronger bond with the oxime than with the enzyme and depart, thus restoring the enzyme's function (Mercey *et al.,* 2012*).*

**Difficulties in Oxime Reactivator Treatment**

Oximes are a critical component to the therapeutic regimen but there are several limitations to oxime therapy which can present significant challenges to clinicians. The first is that many OPs undergo a process called aging, which is the loss of an alkyl R group and the formation of a negative charge on the OP-AChE enzyme complex (Quinn *et al.,* 2017). This

negative charge causes a repulsion between the nucleophilic oxime and the inhibited enzyme, preventing reactivation and forming a completely inactivated enzyme (Antonijevic and Stojilijkovic, 2007). Currently, none of the oxime reactivator platforms can reactivate aged AChE. Substantial differences exist between OPs with respect to half life-times required for aging to occur. Research suggests that aged AChE occurs fastest with soman~2-3minutes (Shafferman *et al.,* 1996; Saxena *et al.,* 1998; de Jong *et al.,* 1984) whereas time of aging varies significantly among sarin, VX, cyclosarin, and OP pesticides (Worek *et al.,* 2004). It is suggested that OPs with branched chain R groups, like isopropyl in sarin and pinacolyl in soman, are more likely to age, and age faster, than those that do not have branched chain R groups, such as the diethylphosphoryl moieties found in VX, VR, CVR and paraoxon (Shafferman *et al.,* 1996; Worek *et al.,* 2004; Worek *et al.,* 2013).

The other concern is that oximes possess varying efficacies towards the different OPs. OPs are a diverse class of compounds and a single broad-spectrum reactivator has yet to be found. Research shows that 2-PAM, TMB-4, obidoxime, HI-6, and HLö-7 are all efficient at reactivating sarin and VX inhibited AChE (Johnson *et al.,* 1970; Sidell *et al.,* 1974; Inns *et al.,* 1983; Maksimovic *et al.,* 1980), but they have varying efficacies with the other major OP nerve agents (i.e., cyclosarin, tabun, soman), with HLö-7 being the most powerful at reactivating soman, sarin, cyclosarin, and tabun (Worek *et al.,* 1998; de Jong *et al.,* 1989; Worek *et al.,* 2005) and MMB-4 being one of the poorest overall (Aurbek *et al.,* 2006; Bartling *et al.,* 2007). The current oximes vary in their efficacies with OP insecticides as well, with 2-PAM being moderate to poor against insecticidal OPs, as are HI-6 and HLö-7, and TMB-4 and obidoxime being the most effective reactivators for OP insecticides (Jokanovic *et al.,* 1995; Worek *et al.,* 1996).

Another problem with these oxime platforms is the inability to cross the blood brain barrier (BBB) to any significant extent and regenerate brain inhibited AChE (Gallagher *et al.,* 2016; Kalasz *et al.,* 2014; Lorke *et al.,* 2008; Sakurada *et al.,* 2003; Clement, 1979), thus brain damage can still occur. Extensive research has been devoted to developing new oxime reactivators that are broad spectrum and neuroprotective such as the substituted phenoxyalkyl pyridinium oximes (Chambers *et al.,* 2013), pro-2PAM (Bodor *et al.,* 1975), monoisonitrosoacetone—MINA (Skovira *et al.,* 2010), and the neutral charged imidazole aldoximes and N-substituted 2-hydroxyiminoacetamides (Rakesh *et al.,* 2011), but nothing has come forth as a replacement for 2-PAM or the other oximes.

**Alternative Medical Strategies in Treating Organophosphates**

Another challenge to clinicians is the potential for a high body load of OP after self ingestion or from a lethal exposure to a nerve agent. A high dose exposure of OPs could potentially mitigate the effectiveness of oxime therapy because of the likelihood that reinhibition of the enzyme can occur, thus potentially requiring higher oxime doses that may not be well tolerated in humans (Thiermann *et al.,* 1999). Various medical countermeasures have come forth to act as adjuncts to the current OP therapy. These treatment strategies consist of reversible cholinesterase inhibitors and bioscavenger enzymes. The purpose of these is to improve CNS AChE protection and to scavenge OP molecules in the circulation before the OPs have a chance to reach critical target areas. These countermeasures can be very helpful as they reduce the 'body load' of OP, which can prevent reinhibition of reactivated enzyme. Several of these strategies investigated are given prophylactically, which means they are administered before exposure to the OP. While not applicable towards civilians exposed accidentally, by terrorist attack or by attempted suicide, these can be of value for our warfighters.

## Reversible Cholinesterase Inhibitors

Delivery of pyridostigmine bromide prophylactically in conjunction with posttreatment atropine and 2-PAM has emerged as an effective strategy in treating soman and sarin exposure (Leadbeater *et al.,* 1985). Pyridostigmine bromide is a carbamate compound that reversibly inhibits AChE in the periphery. By inhibiting AChE before OP exposure, some of the AChE is shielded from irreversible inhibition by the OP. Additionally, since soman-inhibited AChE ages very quickly, this treatment strategy has emerged as a way to circumvent that process. However, very little pyridostigmine bromide enters the brain, leaving CNS AChE susceptible to OP-induced inhibition with the potential for brain damage to occur. Other reversible AChE inhibitors have been brought forth that can more effectively cross the blood brain barrier, such as: huperzine A, tacrine, physostigmine, and galantamine (Deshpande *et al.,* 1986; Grunwald *et al.,* 1994; Fricke *et al.,* 1994; Lallement *et al.,* 2002; Albuquerque *et al.,* 2006). While promising, CNS side effects of these drugs have been noted and delivery of a drug prophylactically may not always be feasible.

## Use of Bio-Scavengers to Treat Organophosphates

Another strategy that has been investigated in OP treatment has explored the capability of improving the detoxication of circulating OP molecules in the blood by bioscavengers, with the hope of diminishing the amount of OP that can reach peripheral and neuronal AChE to cause toxicity. The laboratory of Wolfe and company were the first to come up with the scavenger concept. His work focused on increasing the cholinesterase pool in the blood (i.e., increasing the first line of defense) by administration of fetal bovine serum (FBS) AChE. His work showed protection to high levels of nerve agents in mice that were pretreated with FBS AChE (Wolfe *et al.,* 1987). Other scavengers have been investigated since then that are also able to neutralize

OPs. These scavengers can be enzymes or antibodies that are stoichiometric (i.e., 1 enzyme to 1 OP molecule) or catalytic (enzymes that have a turnover rate; 1 enzyme to multiple OP molecules). The catalytic scavengers are still in their infancy and are not the subject of this manuscript and will be discussed no further.

The most promising stoichiometric scavenger that has been investigated is butyrylcholinesterase (BChE). BChE is a serine hydrolase (EC 3.1.1.8) that is synthesized in the liver. Humans have more total protein BChE than AChE--about 10x the amount of BChE compared to AChE total protein (Lockridge, 2015), with the majority of BChE being found in peripheral tissues like the blood and liver (Manoharan *et al.,* 2007). BChE can be an effective scavenger because of its larger active site that can accommodate a wide variety of ester compounds (Li *et al.,* 2005) and its lack of known physiological role; individuals who have a silent BChE gene live normal, healthy lives but may be more susceptible to the adverse effects of certain compounds like succinylcholine (Doenicke *et al.,* 1963; Liddell *et al.,* 1962). This lack of known physiological role and ability to interact with a variety of toxic esters has led researchers to believe that its role may be to serve as a protector of AChE, since many AChE inhibitors, such as OPs, also inhibit BChE. Inhibition of BChE prevents AChE inhibition from these poisons. Inhibition of BChE by OPs is very fast~$10^{7}$-$10^{9}M^{-1}min^{-1}$ (Masson *et al.,* 2010), thus representing one of the first lines of defense in the blood after OP exposure.

However, at high levels of toxicants, especially of OPs, BChE is saturated and easily overwhelmed; therefore, significant amounts of OP can reach AChE and cause toxicity. To counter this, pre-treatment with exogenous BChE has been an extensive area of research since the 1990s. The US military successfully showed that administration of exogenous BChE (significantly increasing the BChE pool) as a pre-treatment to organophosphates afforded

protection to multiple animal models after exposure to lethal levels of nerve agents (Broomfield *et al.,* 1991; Ashani *et al.,* 1991; Wolfe *et al.,* 1992; Raveh *et al.,* 1993). The US Department of Defense (DoD) has since allocated millions of dollars to synthesize BChE as a prophylactic treatment. It was labeled as an Investigational New Drug by the FDA in 2006 (Lenz *et al.,* 2007) and underwent phase I clinical trials by Baxter Health Corporation (Masson *et al.,* 2010). However, this line of treatment does have a few drawbacks. Since stoichiometric scavengers are proteins that bind one molecule of nerve agent per one molecule of scavenger, the concentration of administered scavenger needs to be high enough to neutralize a large amount of OP molecules (Masson *et al.,* 2010; Ashani and Pistinner, 2004). This dose is quite high, at least 200mg/70kg for soman exposure protection (Ashani and Pistinner, 2004). Moreover, mass production of this enzyme has proven to be a challenge and resulted in a very expensive treatment (Masson *et al.,* 2016), which limits the number of people that can receive it (1kg BChE~5000doses, Geyer *et al.,* 2010; Ashani Y, 2000). This treatment, while potentially having great benefit for first responders or warfighters in an OP contaminated area, is limited in its ability to protect civilians and is more than likely not economically feasible.

While substantial efforts have been devoted to developing recombinant BChE that can be easier to mass synthesize (Zhang *et al.,* 2016; Saxena *et al.,* 2010; Huang *et al.,* 2007; Chilukuri *et al.,* 2005; Chilukuri *et al.,* 2008; Geyer *et al.,* 2010; Parikh *et al.,* 2010; Cerasoli *et al.,* 2005; Cerasoli *et al.,* 2005), replicating the long circulatory half life of human plasma BChE, which is about 11 days (Ostergaard *et al.,* 1988), without eliciting an adverse immune response has proven to be difficult (Rosenberg *et al.,* 2015; Chilukuri *et al.,* 2008; Chilukuri *et al.,* 2008). Having an enzyme with a long circulatory residence time with no immune response that can

rapidly scavenge multiple OPs is highly desirable for a bioscavenger, but such an enzyme has yet to be found.

## Pseudo-Catalytic Scavengers

To combat the drawbacks of the stoichiometric scavengers, namely the high amounts of enzyme needed, costs, and required advance knowledge of the attack, oxime reactivators have been under investigation as potential tools to constantly regenerate inhibited BChE and allow for consistent binding and degradation of inhibitor (Nachon *et al.,* 2013; Aurbek *et al.,* 2009), thus providing a pseudo-catalytic, or oxime-assisted catalytic, function. BChE binds to the OP in a process that destroys the OP and leaves the enzyme inhibited because of slow water molecule activation to facilitate the hydrolysis of the OP. Oximes can remove the OP from the inhibited enzyme and allow the newly regenerated enzyme to bind to additional OP molecules. This constant inhibition and regeneration of BChE should mitigate severe signs of toxicity by destroying significant amounts of OP in the circulation, thus affording protection to cardiac, skeletal, and neuronal AChE by decreasing the amounts of OP reaching these targets. Oxime-assisted catalysis with current and developmental oxime platforms for BChE has shown mixed results (Radic *et al.,* 2013; Katalinic *et al.,* 2016; Busic *et al.,* 2016; Konickx *et al.,* 2013; Aurbek *et al.,* 2009). Having an antidote that can be effective against a wide variety of OPs and have diverse therapeutic functions that is low cost would be of great benefit to medical and military personnel.

## Research Summary

Our laboratory has synthesized a series of novel phenoxyalkyl pyridinium oximes (US Patent number: 9,227,937). These oximes, designed by the late Dr. Howard Chambers of Mississippi State University, were synthesized with the intention as potential brain penetrating

AChE reactivators, which the current oxime platforms cannot do. These oximes have added lipophilic moieties which should allow for increased entry into the brain. Previous research completed in our laboratory has shown that these oximes have an ability to reactivate brain AChE *in vivo* after inhibition by sarin and VX surrogates (Chambers *et al.*, 2013; Chambers *et al.*, 2016), increase survivability and attenuate seizure-like behavior after exposure to sarin and VX surrogates when compared to 2-PAM (Chambers *et al.*, 2016), and attenuate OP induced brain damage after exposure to paraoxon and sarin and VX surrogates (Pringle *et al.*, 2018; Dail *et al.*, 2019). The research herein investigates these oximes as potential BChE reactivators with the intention of identifying potential alternative oxime therapeutic mechanisms that may be contributing to the increased survivability seen *in vivo.* In addition to this, intranasal delivery of these oximes was investigated as a potential route of oxime administration to increase the amount of oxime that can enter the brain and reactivate brain AChE.

BChE reactivation by oximes would allow for additional scavenging of OP molecules in the circulation, thus potentially limiting the amount of OP molecules that can reach critical cardiac and neuronal AChE and cause toxicity. Since there is no blood  brain barrier (BBB) present at the olfactory bulb that can restrict oxime access, intranasal delivery would allow for a high amount of drug that can be delivered into the brain and potentially allow for a rapid therapeutic onset.

Chapter 2 explores the ability of several of our novel oximes (OX1, OX9, OX12, OX13, OX15, OX20, OX55, and OX62), in addition to the current oxime platforms used by various nation states (2-PAM, TMB-4, MMB-4, obidoxime (OBD), and HI-6), to reactivate rat, human, and guinea pig serum BChE *in vitro* after exposure to nitrophenyl isopropyl methylphosphonate (NIMP; sarin surrogate), phthalimidyl isopropyl methylphosphonate (PIMP; sarin surrogate),

nitrophenyl ethyl methylphosphonate (NEMP; VX surrogate), nitrophenyl cyclohexyl methylphosphonate (NCMP; cyclosarin surrogate), diisopropyl methyl phosphonate (DFP; commonly used sarin surrogate), as well as the insecticidal oxons paraoxon (PXN; metabolite of parathion), phorate oxon and phorate oxon sulfoxide (PHO and PHOsox; metabolites of phorate). The surrogates used in this study are representatives of some of the most relevant nerve agents (sarin, VX, and cyclosarin) but are safer to work with while still displaying similar potency patterns on AChE compared to the actual nerve agents (Coban *et al.*, 2016). The benefit of these surrogates is that they inhibit AChE and BChE with the same chemical moiety as the 'parent' nerve agents do (Meek *et al.*, 2012; Ohta *et al.*, 2006; Fukuto and Metcalf, 1959). The use of insecticidal oxons serves to identify any broad spectrum efficacy capabilities that our oximes possess against both nerve agent and insecticidal chemistries. Parathion and phorate are toxic OP insecticides which could be used as potential alternative chemical agents in a terrorist scenario if nerve agents are unavailable (Bajgar *et al.*, 2015). The hypothesis for this project is that our novel oximes are more effective BChE reactivators than the current pyridinium and bis-pyridinium oximes.

In chapter 3, oxime reactivation kinetics were performed with the lead oximes from chapter 2 on rat and human serum BChE after inhibition by the sarin surrogate NIMP and paraoxon, the insecticidal metabolite of parathion. Reactivation kinetics produces valuable parameters of oxime effectiveness, such as the affinity and overall reactivity of the oxime for the inhibited enzyme. These measures of affinity and reactivity can provide a quantitative assessment on the effectiveness of an oxime before being tested *in vivo.* Since it is unethical to test the efficacy of oximes in humans, animal models used for *in vitro* and *in vivo* testing are invaluable tools for extrapolation of oxime efficacy to humans. Species differences are observed

for the reactivation potential of oximes and the inhibitory potential of OPs on AChE (de Jong *et al.,* 1984; Worek *et al.,* 2002; Worek *et al.,* 2011; Luo *et al.,* 2007). Therefore, the use of the rat, which is the most common animal used in toxicity testing, was used in addition to human serum to determine if any species related differences in BChE reactivation are present.

In chapter 4, intranasal delivery of novel oxime 20 and 2-PAM was tested as a potential route of oxime administration to decrease OP induced brain AChE inhibition. One of the critical setbacks of the current oxime platforms is the inability for them to cross the blood brain barrier to any significant extent. Sakurada *et al.,* 2003, demonstrated that 10% of 2-PAM administered can pass through the blood brain barrier and potentially reactivate brain AChE; however, this study only investigated 2-PAM alone treated animals. It is likely that in the presence of an OP the amount of 2-PAM that could enter into the brain would be significantly less as most of the 2-PAM would react with inhibited AChE in the periphery. Additionally, it is unclear if 10% is enough to attenuate OP induced brain AChE inhibition and neurotoxicity. The lack of blood brain barrier and the high vascularity in the nasal cavity should allow for entry of a high amount of drug that can rapidly interact with the brain's inhibited AChE, potentially mitigating CNS neurotoxicity. An intranasally administered oxime, in conjunction with a peripherally administered oxime, could produce system wide AChE protection.

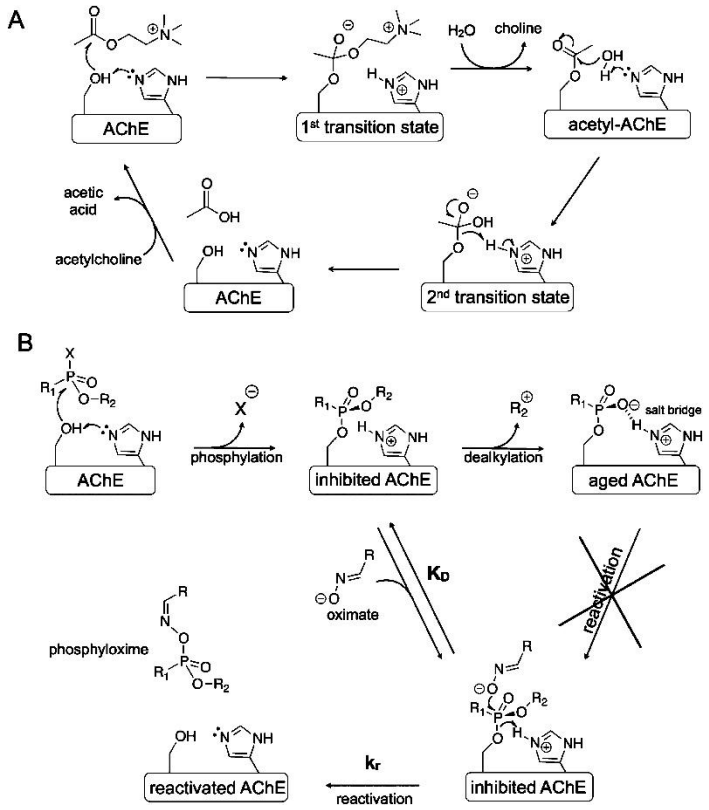


Figure 1.1 A) Mechanism of acetylcholine hydrolysis by AChE. (B) Mechanism of AChE inhibition by organophosphorus nerve agents, aging, and reactivation by oximes. Retrieved from Mercey *et al.*, 2012.

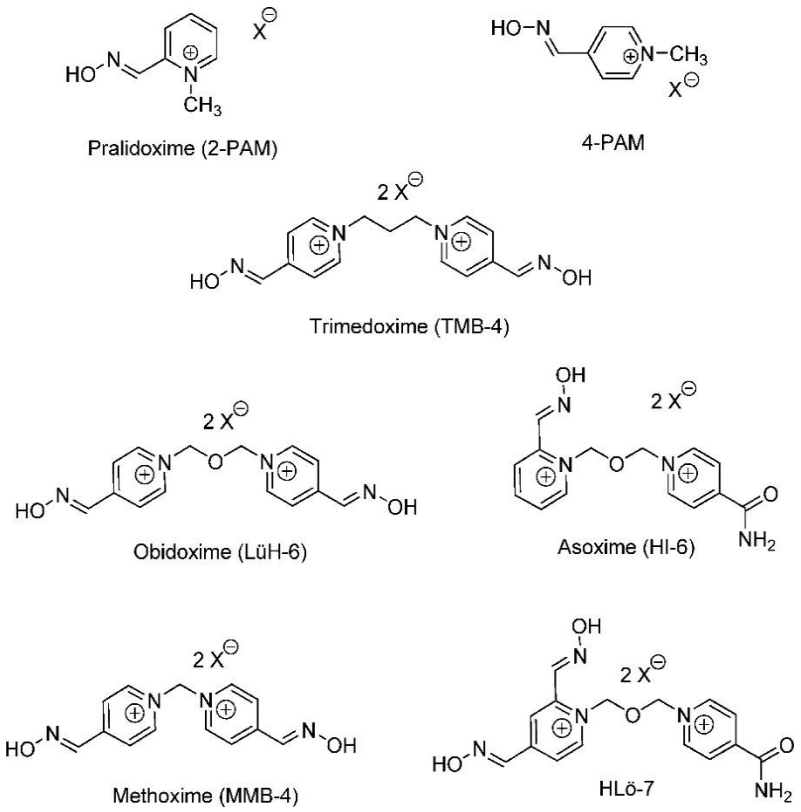


Figure 1.2 Chemical structures of currently used pyridinium and bis-pyridinium oxime reactivators. Retrieved from Mercey *et al.*, 2012.

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CHAPTER II

*IN VITRO* REACTIVATION OF RAT, HUMAN, AND GUINEA PIG SERUM BUTYRYLCHOLINESTERASE BY NOVEL PHENOXYALKYL PYRIDINIUM OXIMES AFTER INHIBITION BY ORGANOPHOSPHORUS COMPOUNDS

## Introduction

Organophosphorus compounds (OPs), such as chemical warfare nerve agents and some insecticides, are highly toxic anticholinesterases that pose a threat to public health because of their high potency, rapid onset of action, and resistance to therapeutics, making them an attractive weapon for rogue nation states and terrorist groups. These compounds persistently inhibit acetylcholinesterase (AChE) by phosphorylation of the catalytic serine hydroxyl group, preventing its function of hydrolyzing the neurotransmitter acetylcholine, an important chemical messenger in the peripheral and central nervous systems that governs critical skeletal, cardiac, and brain functions. Inhibition of AChE leads to an accumulation of acetylcholine at neural synapses, resulting in hyper-stimulation of cholinergic receptors throughout the body causing seizures, respiratory distress, and paralysis (Ecobichon *et al.*, 2001*)*. Death may result if the victim is left untreated.

Current methodology in the treatment of OP poisoning is the administration of atropine sulfate, an anti-muscarinic that blocks the uncontrolled stimulation at peripheral muscarinic receptor sites, and an oxime reactivator to regenerate the inhibited enzyme by removing the

phosphate from the active site serine (Mercey *et al.*, 2012). There are a variety of oxime reactivators, namely the monopyridinium oxime 2-PAM and the bis-pyridinium oximes HI-6, TMB-4, MMB-4, and obidoxime (OBD). Regeneration of the inhibited AChE by oximes is a critical part of the therapeutic regimen to attenuate OP toxicity (Eyer, 2003; Kassa, 2002) but the current oxime therapeutics display varying efficacies towards reactivating OPs (Antonijevic and Stojilijkovic, 2007) and there is no single broad spectrum reactivator. The current oximes also possess poor blood brain barrier penetration (Lorke *et al.*, 2008) and therefore do not protect against OP induced CNS damage.

Researchers have since been trying to find alternative ways to treat OP poisoning. A promising approach has been the study of bioscavengers, with a large body of evidence investigating butyrylcholinesterase (BChE) as a novel therapeutic target. BChE is a serine hydrolase that interacts with a variety of endogenous and exogenous esters, including OPs (Lockridge, 2015). It is this promiscuity that makes it an attractive pharmacological target, as well as its large distribution throughout the body (Manoharan *et al.*, 2007) and its lack of known physiological role (Masson *et al.*, 2010). Interaction of BChE with OPs causes destruction of both substrate and enzyme in a 1:1 fashion (1 molecule of OP is destroyed by 1 molecule of BChE) and prevents the OP from reaching AChE. This stoichiometric process can be easily overwhelmed by high levels of toxicant but pretreatment with exogenous BChE from various sources, thus greatly increasing the levels of BChE in the circulation, has proven to be an effective modality at preventing OP toxicity and mortality in a variety of animal models (Lenz *et al.*, 2007). The major disadvantages to such a treatment are required knowledge of nerve agent attack, the high amount of enzyme needed for protection (Masson *et al.*, 2016; Ashani *et al.*, 2004), technological limitations for mass production of the enzyme (Saxena *et al.*, 2010; Geyer

*et al.*, 2010), and the cost associated with the treatment (Masson *et al.*, 2016). To counteract these limitations, many researchers have focused on using current and developmental oxime reactivators to constantly regenerate inhibited BChE, thus providing a pseudo-catalytic or oxime assisted catalysis (Katalinic *et al.*, 2016; Busic *et al.*, 2016; Radic *et al.*, 2013; Konickx *et al.*, 2013; Aurbek *et al.*, 2009). Inhibition of BChE by an OP in the serum followed by reactivation would allow numerous regenerations of BChE and increase the amount of OP molecules that can be destroyed, potentially decreasing significant amounts of OP that can reach cardiac and neuronal AChE. This treatment would be a more cost effective solution and could potentially be of great value to medical and military personnel.

The current *in vitro* investigation was designed to determine the BChE reactivation potential of 8 novel phenoxyalkyl pyridinium oximes (synthesized by the late Dr. Howard Chambers) and the current oxime platforms 2-pyridinium aldoxime (2-PAM), obidoxime (OBD), asoxime (HI-6), methoxime (MMB-4), and trimedoxime (TMB-4) in rat, human, and guinea pig serum after exposure to 8 OP compounds that consisted of nerve agent and insecticidal chemistries. Rat serum was chosen as a source of BChE as it is a common model used in toxicity testing and is the model used at Mississippi State University; guinea pig serum was used as a source of BChE because it is a common model used in nerve agent testing; and human serum was used for translational purposes and to observe any differences among the tested animal species. Since Mississippi State University is not allowed to work with the actual nerve agents, our laboratories have synthesized surrogates for some of the main nerve agents (Meek *et al.*, 2012). These surrogates inhibit AChE and BChE with the same chemical moieties as the actual nerve agents but are safer to work with and are thus ideal for initial therapeutic studies. The nerve agent OPs that were tested consisted of nitrophenyl isopropyl methyl phosphonate

(NIMP; a sarin surrogate), phthalimidyl isopropyl methylphosphonate (PIMP; a sarin surrogate), nitrophenyl ethyl methylphosphonate (NEMP; a VX surrogate), nitrophenyl cyclohexyl methyl phosphonate (NCMP; a cyclosarin surrogate), and diisopropylfluorophosphate (DFP; a commonly used surrogate for nerve agent testing). In addition to this, two insecticidal OPs: paraoxon (PXN; metabolite of parathion) and phorate oxon/phorate oxon sulfoxide (PHO, PHOsox; metabolites of phorate) were also tested.

The data produced demonstrated proof of principle that our novel oximes can reactivate BChE more effectively than the current oxime therapeutics. The use of different animal models will contribute to building a library of oxime reactivation data within these species so that, if necessary, future modifications can be made towards the oximes (structural modifications/dosages) in addition to identifying differences in reactivation among different animal species for translational purposes.

## Materials and Methods

### Materials

#### *Organophosphorus Compounds*

Phthalimidyl isopropyl methylphosphonate (PIMP; sarin surrogate), nitrophenyl isopropyl methylphosphonate (NIMP; sarin surrogate), nitrophenyl ethyl methylphosphonate (NEMP, VX surrogate), nitrophenyl cyclohexyl methylphosphonate (NCMP; cyclosarin surrogate) were synthesized in our laboratory and by others (Meek *et al.*, 2012; Ohta *et al.*, 2006; Fukuto and Metcalf, 1959). Paraoxon and diisopropylfluorophosphate (DFP) were purchased from Sigma-Aldrich while phorate oxon and phorate oxon sulfoxide were purchased from Chem Services (see Figure 2-1 for OP structures).

### *Oxime Reactivators*

Eight novel oximes (OX1, OX9, OX12, OX13, OX15, OX20, OX55, and OX62), synthesized by the late Dr. Howard Chambers and described in Chambers *et al.*, 2013, were screened in the in vitro studies (see Figure 2-2 for oxime structures and substitutions). These oximes contained various substitutions on the phenoxy ring and different alkyl lengths. Oxime 13 was the only oxime tested with a linker chain of 3 alkyl groups, while four oximes (oxime 1, 12, 20 and 55) contained 4 alkyl groups in their linker chains, and three oximes (oxime 9, 15, and 62) contained five alkyl groups in their linker chains. The current oxime therapeutics: 2-pyridinium aldoxime (2-PAM), asoxime (HI-6), methoxime (MMB-4), obidoxime (OBD), and trimedoxime (TMB-4) were purchased from Sigma-Aldrich (see Figure 2-3 for structures of the current oxime therapeutics).

### *Tissue*

Pooled human serum, Hartley guinea pig serum, and Sprague Dawley rat serum were used as sources of BChE and were purchased from Innovative Research, Novi, MI.

## Methods

### *Determination of Butyrylcholinesterase Inhibition*

A modification of the Ellman method was used to determine BChE activities. A discontinuous spectrophotometric assay was performed using butyrylthiocholine (BTCh) as the substrate and 5,5'-dithio-bis(nitrobenzoic acid) (DTNB) as the chromogen (Ellman *et al.*, 1961; Chambers *et al.*, 1988).

Pooled rat (Sprague Dawley), human, or guinea pig (Hartley) serum was diluted to appropriate assay concentrations in 1.5ml test tubes: rat serum was diluted to 0.250ml serum/ml, guinea pig serum was diluted to 0.050ml serum/ml, and human serum was diluted to 0.025 serum/ml in 0.05M Tris-HCl buffer, pH 7.4 (25˚C). Treatment groups consisted of EtOH (vehicle control, FC 1%) and various concentrations of OP (in EtOH). The vehicle control group was used to determine total BChE enzyme activity and to make sure the vehicles themselves did not cause any inhibition. The various OP concentrations determined the total amount of inhibited enzyme. Serum was incubated with 10µl ethanol vehicle (FC 1%) or OP (in EtOH) in a shaking water bath (37°C) for 15 min. Following this, 250µl of each sample was added into 9.75ml of ice cold 0.05M Tris-HCl buffer. This dilution step removes excess OP that can re-inhibit the enzyme. After the dilution, 2ml of sample was aliquoted into each of 4 separate glass test tubes. To correct for non-cholinesterase hydrolysis, 20µl of eserine sulfate (in deionized water, FC 10µM) was added to one of the tubes while the other three were used for BChE measurements. These samples were placed in a shaking water bath (37°C) for 15 min. After this, 20µl of BTCh (in EtOH, FC 1mM), was added to all tubes. The samples were incubated for another 15 min. The reaction was then terminated and color developed by adding 250µl of sodium dodecyl sulfate (SDS, FC 0.5%)/DTNB (FC 0.6mM) (4:1) mixture to every tube. Absorbance was measured at 412nm in a Thermo Scientific Biomate 3 spectrophotometer. Concentrations of OP that inhibited around 85-90% of BChE activity were used.

### *Calculation of Butyrylcholinesterase Inhibition*

Total BChE inhibition was calculated first by determining the average activity of the three sub- samples and subtracting the eserine value. Total percentage inhibition was calculated from the absorbances of the total tissue control and total inhibition control (OP alone treatment

group) (equation 1). Concentrations of OP that inhibited about 80% of BChE activity were used for the reactivation experiments.

Equation 1:

$$Inhibition_{total}\ \% = \frac{(Vehicle\ Control) - (OP\ Alone)}{(Vehicle\ Control)} * 100 \quad (2.1)$$

***Determination of Butyrylcholinesterase Reactivation***

Pooled rat (Sprague Dawley), human, or guinea pig (Hartley) serum was diluted to appropriate assay concentrations in 1.5ml test tubes: rat serum was diluted to 0.250ml serum/ml, guinea pig serum was diluted to 0.050ml serum/ml, and human serum was diluted to 0.025 serum/ml in 0.05M Tris-HCl buffer, pH 7.4 (25˚C).Treatment groups were: 1) EtOH:DMSO (vehicle control, 1:1 ratio of EtOH and DMSO, 1%v/v), 2) OP:DMSO (OP alone), 3) EtOH:Oxime (Oxime alone), 4) OP:Oxime (Oxime+OP). The vehicle control group was used to determine total BChE enzyme activity and to make sure the vehicles themselves did not cause any inhibition. The OP alone group determined the total inhibited enzyme, the oxime alone group determined if the oxime displayed any inhibitory activity towards the enzyme, and the OP+Oxime group determined the total amount of reactivated enzyme.

Serum was incubated with 10µl ethanol vehicle (FC 1%) or OP (in EtOH) in a shaking water bath (37°C) for 15 min. Following this, the oxime (FC 100µM) was delivered in a 1:1 solution of dimethyl sulfoxide (DMSO) and ethanol and incubated for 30 min to allow for reactivation. Following this, 250µl of each sample was added into 9.75ml of ice cold 0.05M Tris-HCl buffer. This dilution step removes excess OP that can re-inhibit the enzyme. After the

dilution, 2ml of sample was aliquoted into each of 4 separate glass test tubes. To correct for non-cholinesterase hydrolysis, 20µl of eserine sulfate (in deionized water, FC 10µM) was added to one of the tubes while the other three were used for BChE measurements. These samples were placed in a shaking water bath (37°C) for 15 min. After this, 20µl BTCh (in EtOH, FC 1mM) was added to all tubes. The samples were incubated for another 15 min. The reaction was then terminated and color developed by adding 250µl of sodium dodecyl sulfate (SDS, FC 0.5%)/DTNB (FC 0.6mM) (4:1) mixture to every tube. Absorbance was measured at 412nm in a Thermo Scientific Biomate 3 spectrophotometer and three replications were completed for each oxime.

### *Calculation of Butyrylcholinesterase Reactivation*

To determine the amount of BChE reactivation, the percent of inhibited enzyme was calculated first. This was accomplished by calculating the average activity of the three sub-samples and subtracting the eserine value. After this, total percentage inhibition was calculated from the absorbances of the total tissue control and total inhibition control (OP alone treatment group) (equation 2.2).

$$Inhibition_{total}\ \% = \frac{(Vehicle\ Control) - (OP\ Alone)}{(Vehicle\ Control)} * 100 \qquad (2.2)$$

Next, the inhibition of the oxime control and oxime+OP groups were calculated using equation 2.3.

$$Inhibition_{sample}\ \% = \frac{(Oxime\ Control) - (Oxime + OP\ Sample)}{(Oxime\ Control)} * 100 \qquad (2.3)$$

Finally, the total reactivation percentage for each sample was calculated by subtracting the oxime+OP inhibition from total inhibited enzyme activity which yielded activity of reactivated enzyme (equation 2.4).

$$Reactivation\ \% = \frac{Inhibition_{total} - Inhibition_{sample}}{Inhibition_{total}} * 100 \quad (2.4)$$

***Statistical Analysis***

The effect of oximes on percent reactivation was assessed using an analysis of variance (ANOVA) with PROC MIXED in SAS for Windows v9.4 (SAS Institute, Inc., Cary, NC). Separate models were run for each inhibitor for human, rat, and guinea pig data. In the case of a significant oxime effect, an LSMESTIMATE statement was used to compare the means for each novel oxime to each of the current antidotes (2-PAM, MMB-4, TMB-4, OBD, HI-6). The SIMULATE option was used to adjust p-values for multiple comparisons for each set of pairwise comparisons. The distribution of residuals was evaluated to assess if the assumptions of normality and homoscedasticity had been met. The data were as determined to be normally distributed by visual assessment of the residual plots. An alpha level of 0.05 was used to determine statistical significance.

**Results**

Our novel phenoxyalkyl pyridinium oximes proved to be more efficient BChE reactivators for several OP compounds for rat inhibited BChE when compared to 2-PAM and the bis-pyridinium oximes HI-6, TMB-4, MMB-4, and obidoxime (OBD). For PIMP and NEMP - inhibited rat BChE, reactivation by our novel oximes ranged from 46-78% for PIMP and 18-88% for NEMP while 2-PAM and the bis-pyridinium oximes ranged from 31-47% for PIMP and 0-

8% for NEMP (Figure 2-4). For paraoxon-inhibited rat BChE, our novel oximes displayed reactivation ranges from 33-95% while 2-PAM and the bis-pyridinium oximes exhibited reactivation ranges from 4-42% (Figure 2-5). For phorate oxon and phorate oxon sulfoxide inhibited rat BChE, 2-PAM proved to be the more efficient BChE reactivator. All oximes displayed greater BChE reactivation towards phorate oxon sulfoxide compared to phorate oxon (Figure 2-6). For NIMP and DFP-inhibited rat BChE reactivation by our novel oximes ranged from 38-82% wile 2-PAM and the bis-pyridinium oximes ranged from 0-46% (Figure 2-7). Reactivation of NCMP-inhibited rat BChE demonstrated poor reactivation by all oximes tested (Figure 2-8). Novel oxime 15 proved to be the most effective and broad spectrum acting reactivator for rat OP-inhibited BChE.

In general, all oximes were poor reactivators of OP-inhibited guinea pig BChE with the exception of PIMP-inhibited BChE (37-69% for the novel oximes and 34-58% for 2-PAM and the bis-pyridinium oximes, Figure 2-9).

For OP-inhibited human BChE, the only effective novel oxime was oxime 20. Oxime 20 displayed significant reactivation efficacy with PIMP-inhibited BChE (79%, Figure 2-14), NEMP-inhibited BChE (70%, Figure 2-14), paraoxon-inhibited BChE (66%, Figure 2-15), phorate oxon-inhibited BChE (83%, Figure 2-16), phorate oxon sulfoxide-inhibited BChE (96%, Figure 2-16), and NCMP-inhibited BChE (65%, Figure 2-18). 2-PAM and the bis-pyridinium oximes, in general, were not effective human BChE reactivators for most of the OP compounds tested. Exceptions were PIMP (reactivation ranges from 36-66%, Figure 2-14), NIMP (72% for 2-PAM, Figure 2-17), phorate oxon (51% for TMB-4, Figure 2-16) and phorate oxon sulfoxide

(58% for 2-PAM, Figure 2-16). Interestingly, novel oximes 13, 55, and 62 displayed significant BChE inhibition.

## Discussion

Several novel phenoxyalkyl pyridinium oximes, in conjunction with the current oxime in the United States, 2-PAM, and several bis-pyridinium oximes (TMB-4, MMB-4, HI-6, and obidoxime) were tested as potential BChE reactivators after inhibition with several OP compounds which consisted of nerve agent and insecticidal chemistries. The novel oximes varied in the number of their alkyl linker groups and the substitutions on their phenoxy ring. Oxime 13 was the only oxime tested with a linker chain of 3 alkyl groups, while four oximes (oxime 1, 12, 20 and 55) contained 4 alkyl groups in their linker chain, and three oximes (oxime 9, 15, and 62) contained five alkyl groups in their linker chain. The oxime position on our novel oximes is in the para position, which is similar to TMB-4, MMB-4, and obidoxime except for the fact that these bis-pyridinium oximes have an oxime functional group on each pyridinium ring. 2-PAM and HI-6 have a single oxime group in the ortho position.

Substantial species differences in BChE reactivation were observed by all of the oximes tested. In general, all oximes showed higher BChE reactivation for all compounds tested towards OP-inhibited rat BChE. The reactivation efficacy proceeded from rat>human>guinea pig. The only notable difference was novel oxime 20, which showed impressive, broad spectrum reactivation towards 6 of the 8 OP compounds tested for human BChE, reaching greater than 70% reactivation for sarin and VX surrogates, and the phorate metabolites (phorate oxon and phorate oxon sulfoxide). Interestingly, the only OP compound which showed consistent, appreciable reactivation for all oximes in all species was PIMP-inhibited BChE, one of the sarin surrogates tested. The other sarin surrogate, NIMP, and DFP, were not reactivated as effectively

as PIMP-inhibited BChE and, in fact, DFP, in addition to NCMP, the cyclosarin surrogate, proved to be one of the more difficult OPs to reactivate in all species tested. NIMP displayed similar reactivation efficacy in rat compared to PIMP but was different in human and guinea pig. This is an interesting observation since NIMP and PIMP should theoretically display similar reactivation efficacies since the phosphorylated product on the enzyme complex is the same compound: isopropyl methylphosphonate. A similar pattern was observed with paraoxon, phorate oxon, and phorate oxon sulfoxide. All three of these compounds become diethyl phosphates once their respective leaving group departs. Most of the oximes displayed higher reactivation efficacy towards paraoxon-inhibited BChE. Interestingly, the two phorate metabolites, phorate oxon and phorate oxon sulfoxide, produced different reactivation results as well, with an increase of reactivation seen with phorate oxon sulfoxide when compared to phorate oxon.

Even though several of these OP compounds leave the enzyme phosphorylated with similar chemical moieties, differences in their reactivation could be due to their different leaving groups. The leaving group plays an important part in orienting the phosphoryl group in the active site once the OP is bound in the enzyme and has been demonstrated to affect the inhibition, aging, and spontaneous reactivation rates of OP-inhibited AChE (Carr and Chambers, 1996). These differences in orientation could play an important role in positioning the OP within the active site that may orient the R groups on the phosphoryl moiety in a position that could cause steric hindrance and potentially reduce oxime entrance into the active site, especially with compounds that have large R groups. Indeed, the compounds with the larger side chains, NCMP and DFP, proved to be the most challenging OPs to reactivate in all species tested for most of the oximes tested. Interestingly, this was not the case for oxime 20 reactivation of NCMP-inhibited

human BChE. Therefore, this assumption cannot be generalized across all species and for all OPs. It is more likely that the combination of the structure of the phosphoryl moieties and the size/species differences in the enzyme structure are the important determinants of oxime induced reactivation. Research suggests that rat and human BChE exhibit high similarities in their amino acid sequence, but differences between rat BChE and human BChE exist between their inhibition by OPs, reactivation by oximes, and size of their active site (Boeck *et al.,* 2002). The smaller active site in rat BChE could explain the low reactivation seen for all oximes with the OPs that have larger R groups, such as the cyclohexyl found in NCMP and the diisopropyl found in DFP, since these bulky side chains could block the active site.

## Conclusion

The results indicated that several of our novel phenoxyalkyl pyridinium oximes are more effective BChE reactivators than the current oxime therapeutics. Novel oxime 15 was the most effective oxime for rat OP-inhibited BChE while novel oxime 20 was the most effective oxime for human OP-inhibited BChE. Novel oxime 20 did show sufficient reactivation efficacy for OP-inhibited rat BChE. This oxime has emerged as one of our lead *in vivo* oximes. Research in our laboratory has shown increased survivability after exposure to sarin and VX surrogates compared to 2-PAM (Chambers *et al.,* 2016). It is plausible that oxime induced reactivation of OP inhibited BChE in the circulation could be detoxifying significant amounts of OP, thus contributing to increased survival rates.

Both of these oximes exhibited broad spectrum capabilities for nerve agent and insecticidal chemistries. This is promising since it is highly desirable for an oxime to have high efficacy for a scavenging enzyme when it is inhibited by different OPs. This greatly increases the

chances of additional scavenging of OP molecules regardless of the OP and could be used in conjunction with more powerful AChE reactivators to allow for additional protection.

While these experiments demonstrated proof of principle that they can reactivate OP inhibited BChE, several questions remain: such as how long does it take to achieve >50% BChE reactivation, and how efficient are these oximes for OP-inhibited BChE? It is likely the assay reactivation time of 30 minutes used in the present screening assays is too long to be considered an effective BChE reactivator. Reactivation needs to happen quickly as the OP will leave the circulation and enter into the target tissues very rapidly. It is also unclear how much BChE reactivation is needed to detoxify enough OP molecules to reduce toxicity. Nevertheless, these experiments demonstrate promising results that require further investigation.

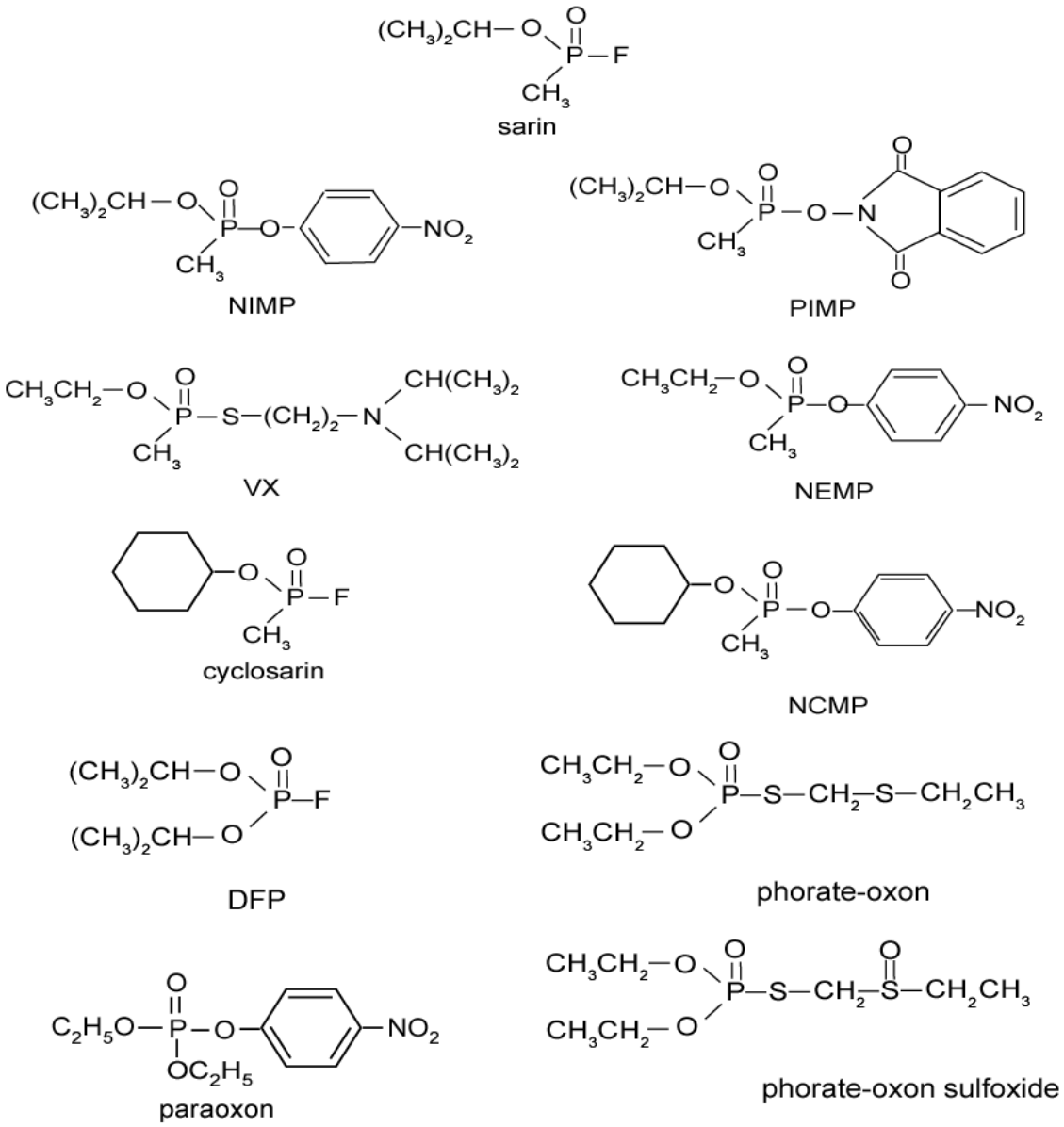
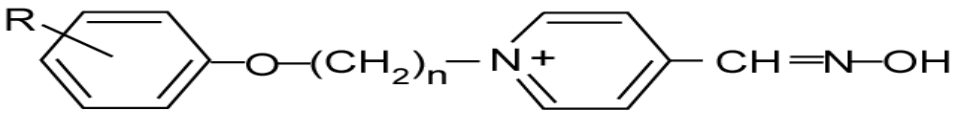



Figure 2.1 Structure of nerve agents, nerve agent surrogates, and OP insecticidal oxons



**Oxime 1 n= 4 R= 4-Cl-**

**Oxime 9 n= 5 R= 4-$CH_3$-O-**

**Oxime 12 n= 4 R= 4-$CH_3CH_2C$(:O)-**

**Oxime 13 n= 3 R= 3-CH=CHCH=CH-4**

**Oxime 15 n= 5 R=4-$CH_3CH_2C$(:O)-**

**Oxime 20 n= 4 R= 4-Ph-$CH_2$-O-**

**Oxime 55 n= 4 R= 4$(CH_3)_3CCH_2C(CH_3)_2$**

**Oxime 62 n= 5 R= 4-$(CH_3)_3CCH_2C(CH_3)_2$**

Figure 2.2 Structure of novel phenoxyalkyl pyridinium oximes (US patent 9,277,937)

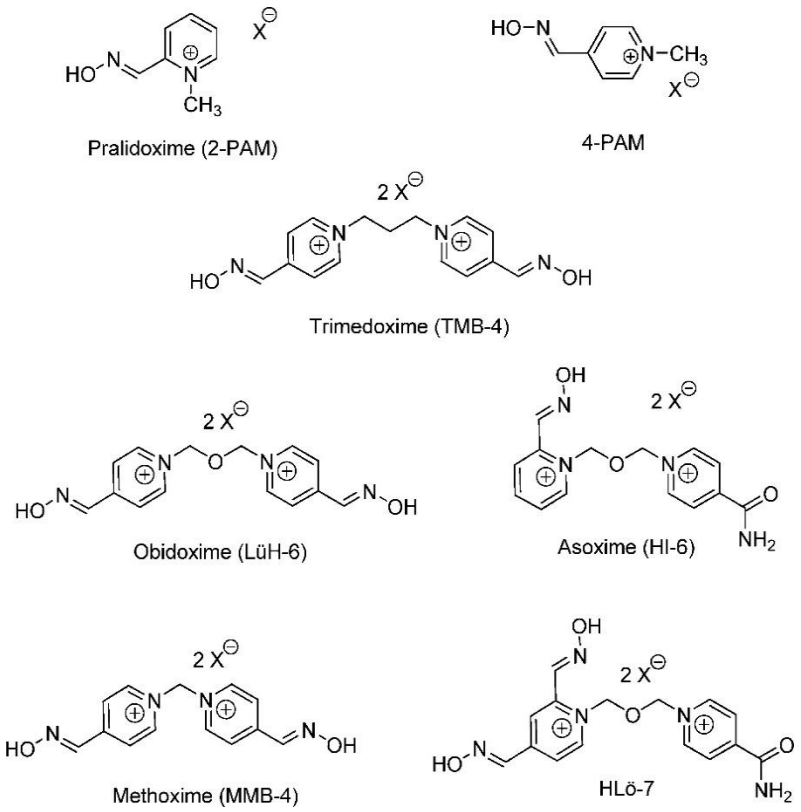



Figure 2.3 Chemical structures of currently used pyridinium and bis-pyridinium oxime reactivators. Retrieved from Mercey *et al.*, 2012.

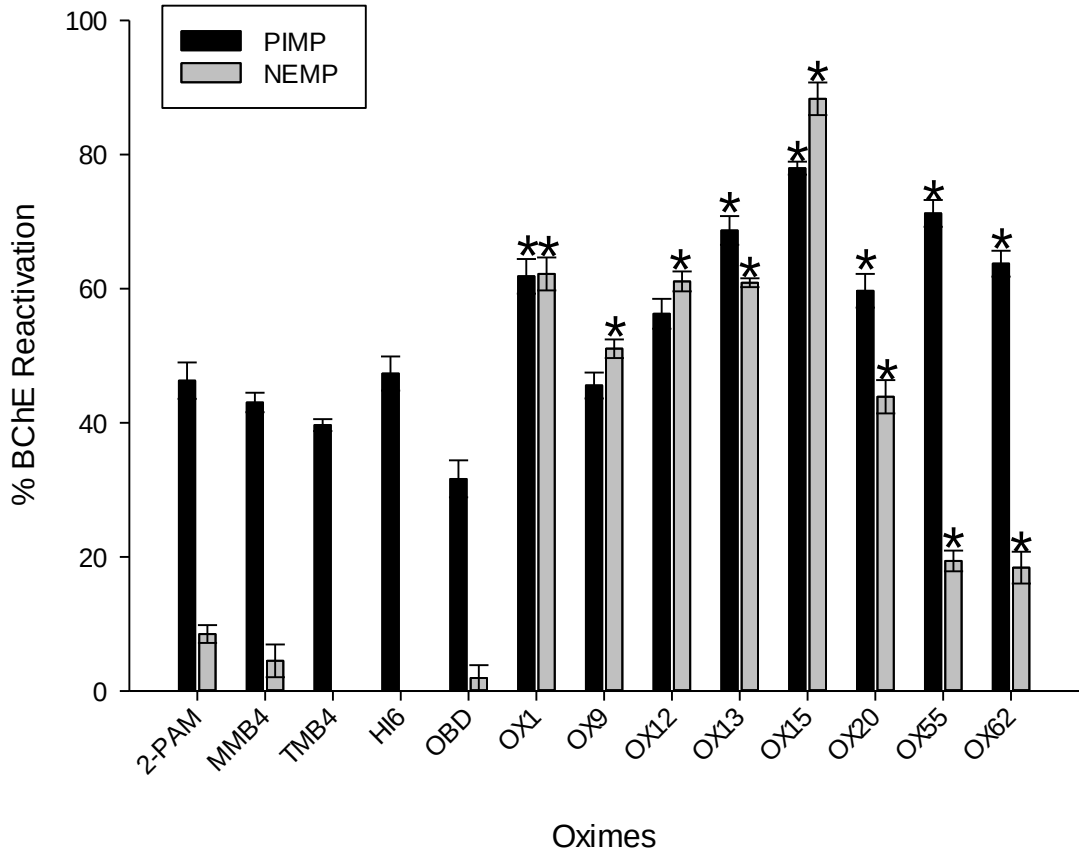



Figure 2.4 Rat serum BChE reactivation after inhibition by PIMP and NEMP. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 p*≤0.05 compared to 2-PAM.

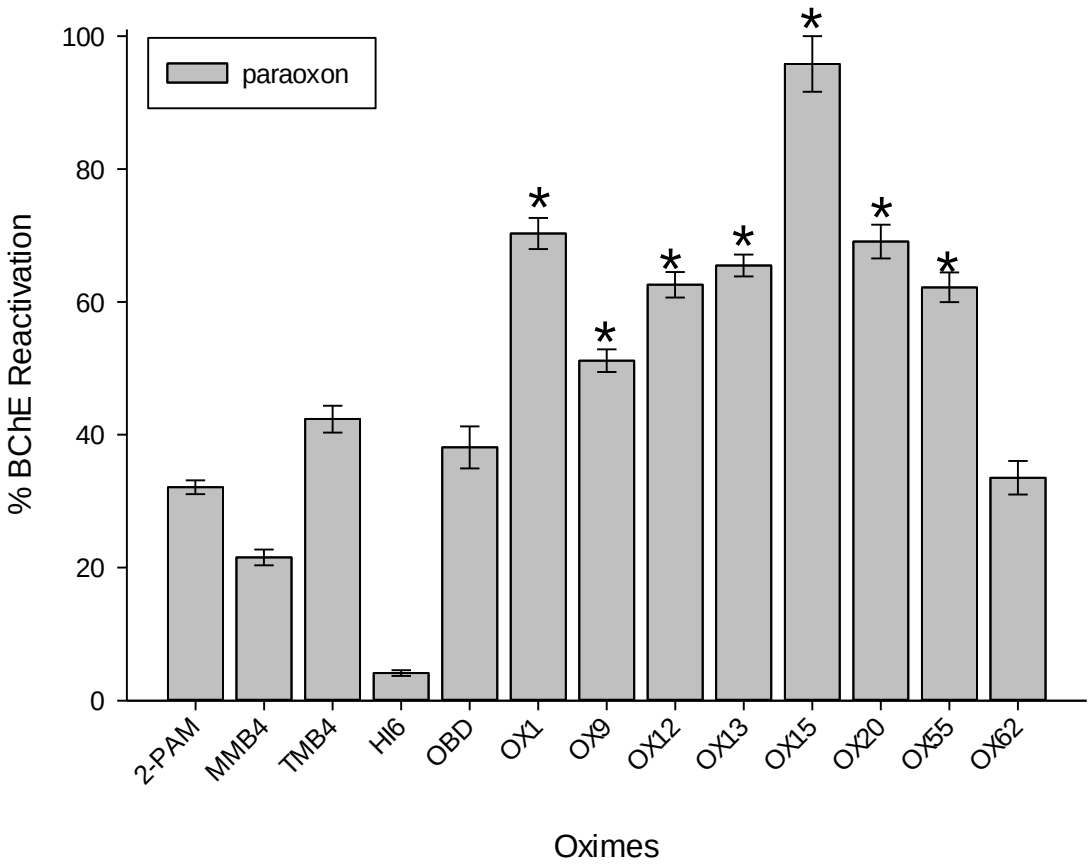



Figure 2.5 Rat serum BChE reactivation after inhibition by paraoxon. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 p*≤0.05 compared to 2-PAM

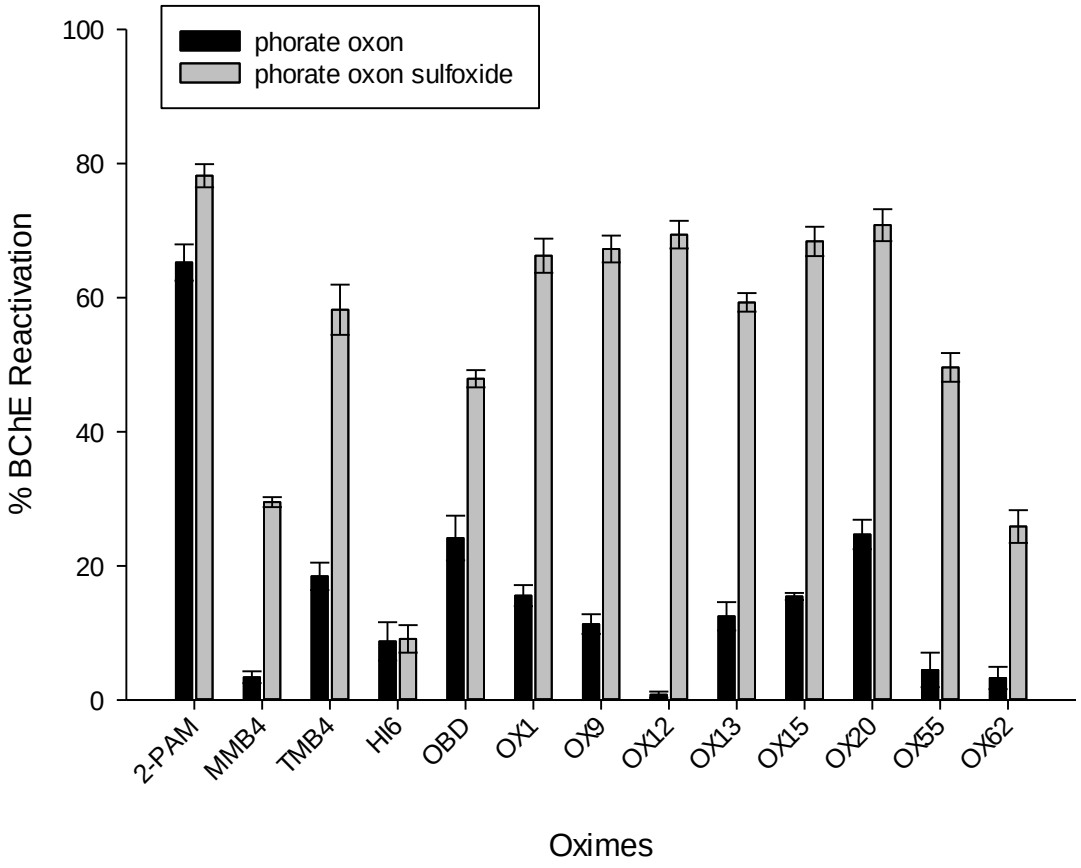



Figure 2.6 Rat serum BChE reactivation after inhibition by phorate oxon and phorate oxon sulfoxide. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3

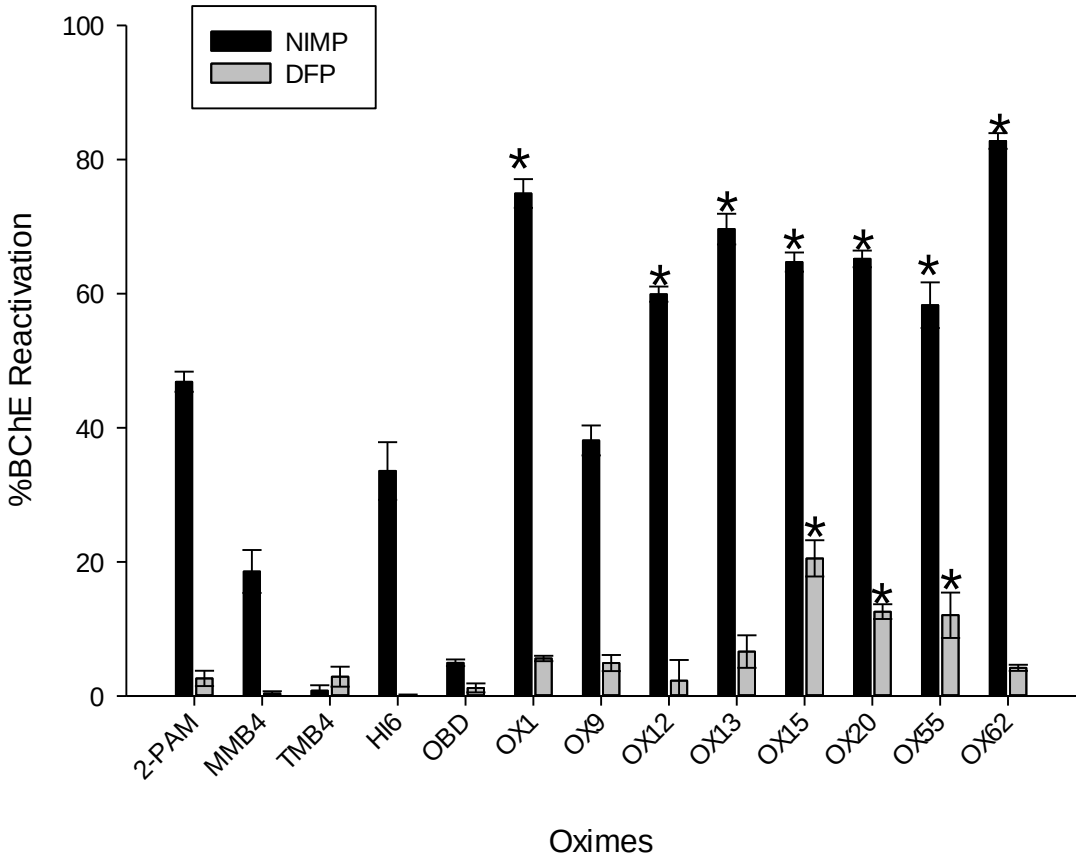



Figure 2.7 Rat serum BChE reactivation after inhibition by NIMP and DFP. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 p*≤0.05 compared to 2-PAM

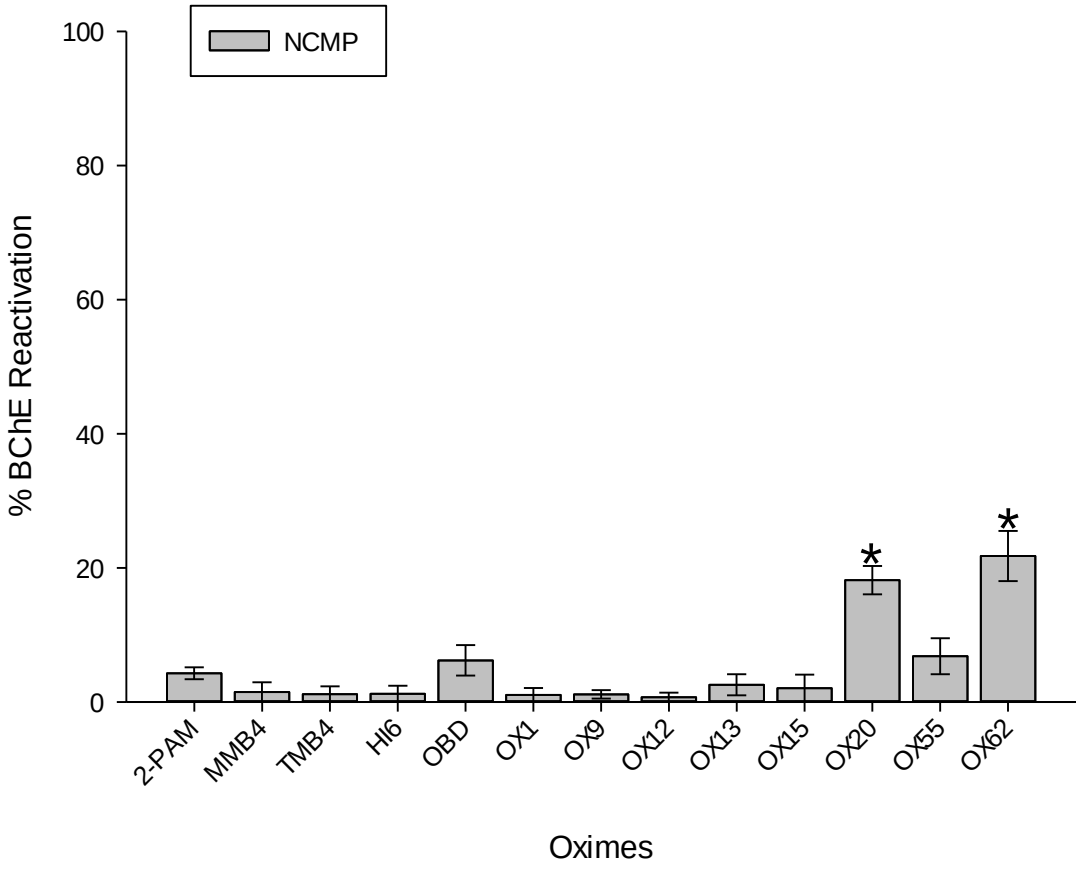



Figure 2.8 Rat serum BChE reactivation after inhibition by NCMP. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 p*≤0.05 compared to 2-PAM

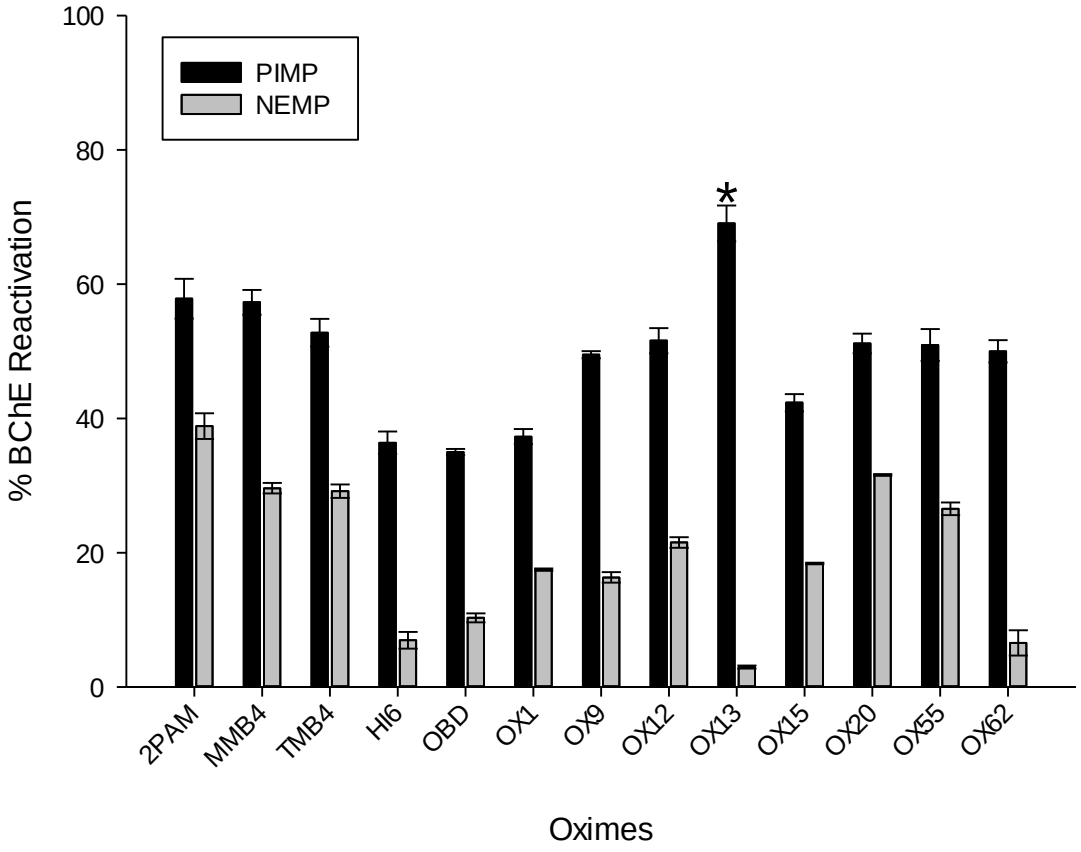



Figure 2.9 Guinea pig serum BChE reactivation after inhibition by PIMP and NEMP. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 $p^* \leq 0.05$ compared to 2-PAM

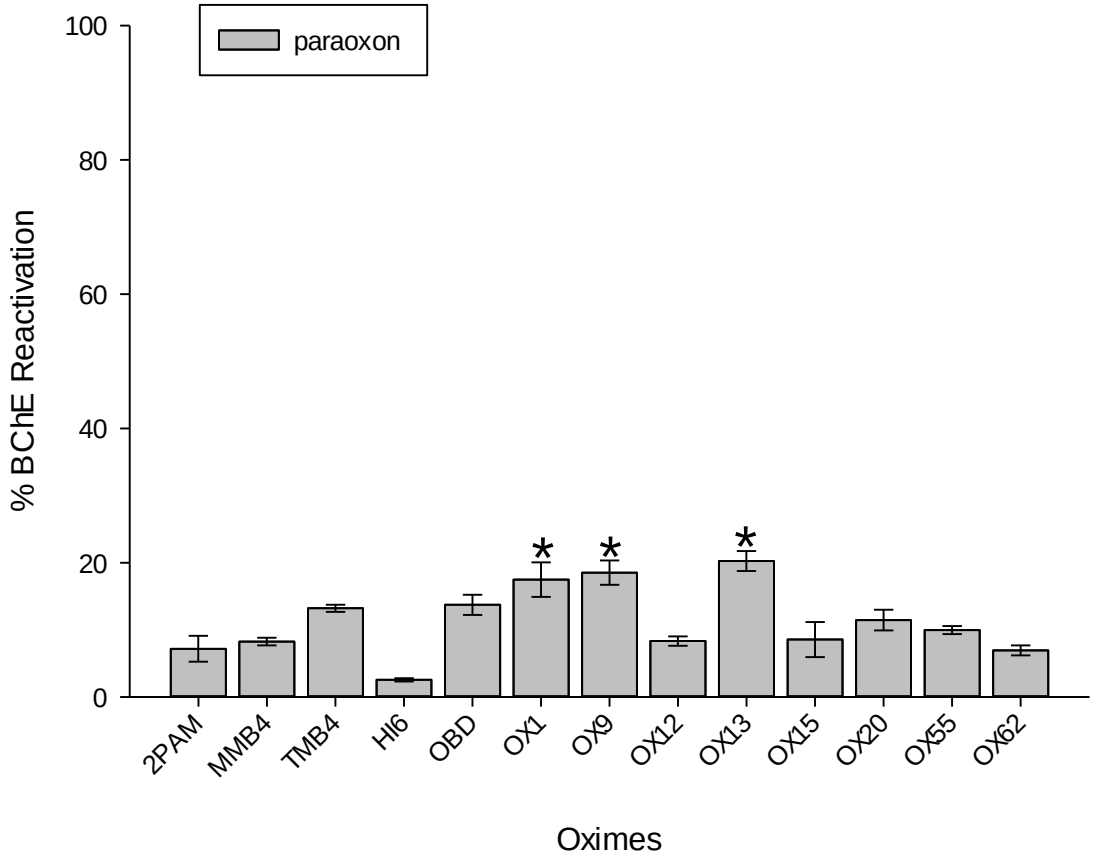



Figure 2.10 Guinea pig serum BChE reactivation after inhibition by paraoxon. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 p*≤0.05 compared to 2-PAM

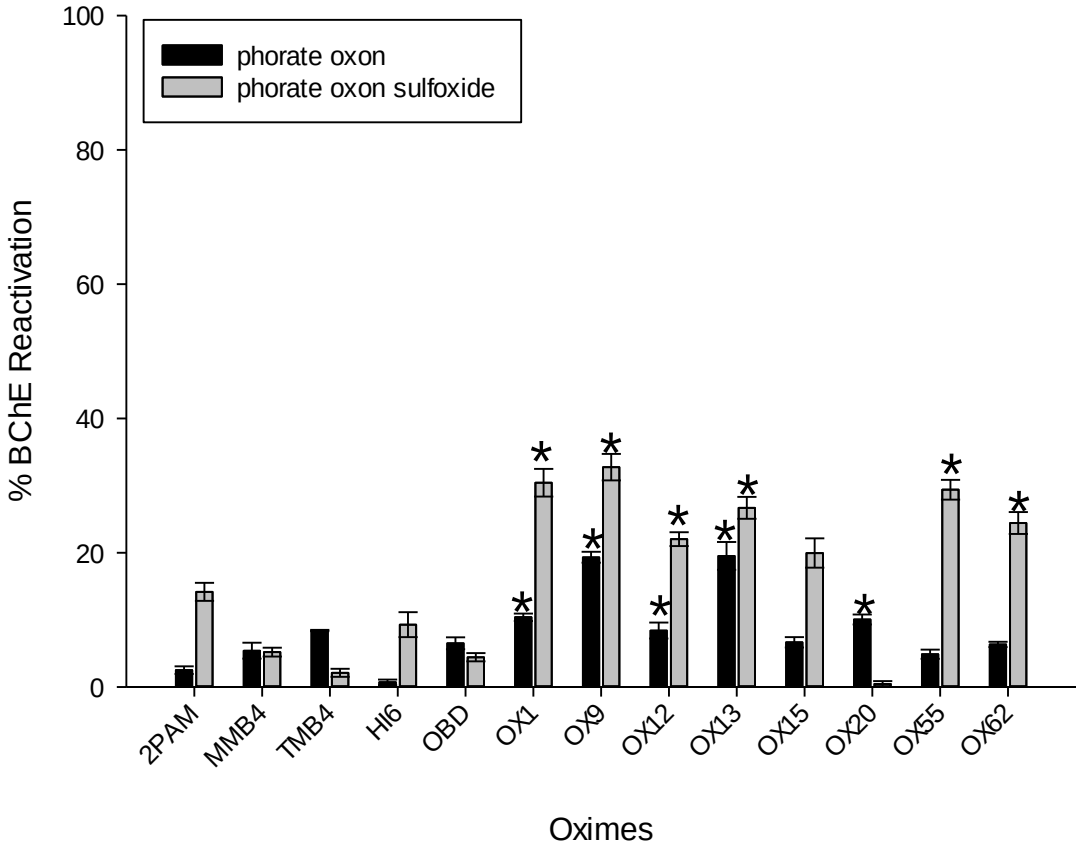



Figure 2.11 Guinea pig serum BChE reactivation after inhibition by phorate oxon and phorate oxon sulfoxide. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 p*≤0.05 compared to 2-PAM

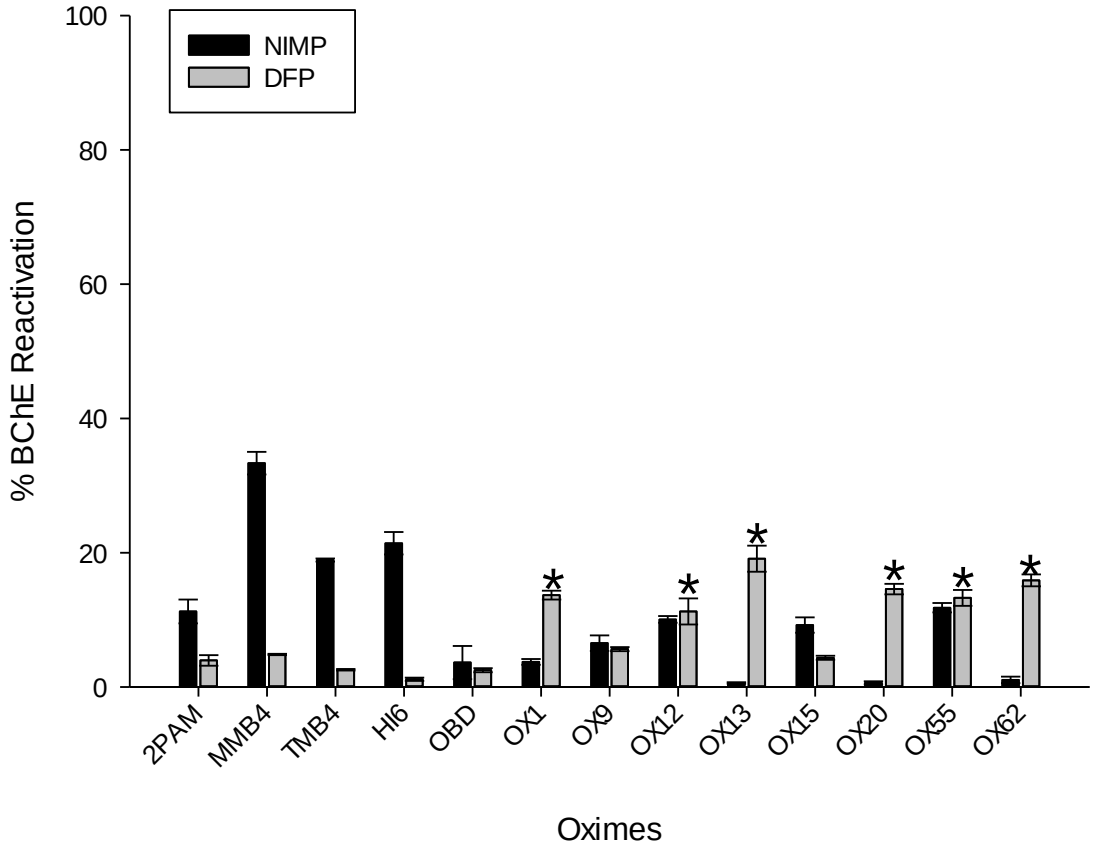



Figure 2.12 Guinea pig serum BChE reactivation after inhibition by NIMP and DFP. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 p*≤0.05 compared to 2-PAM

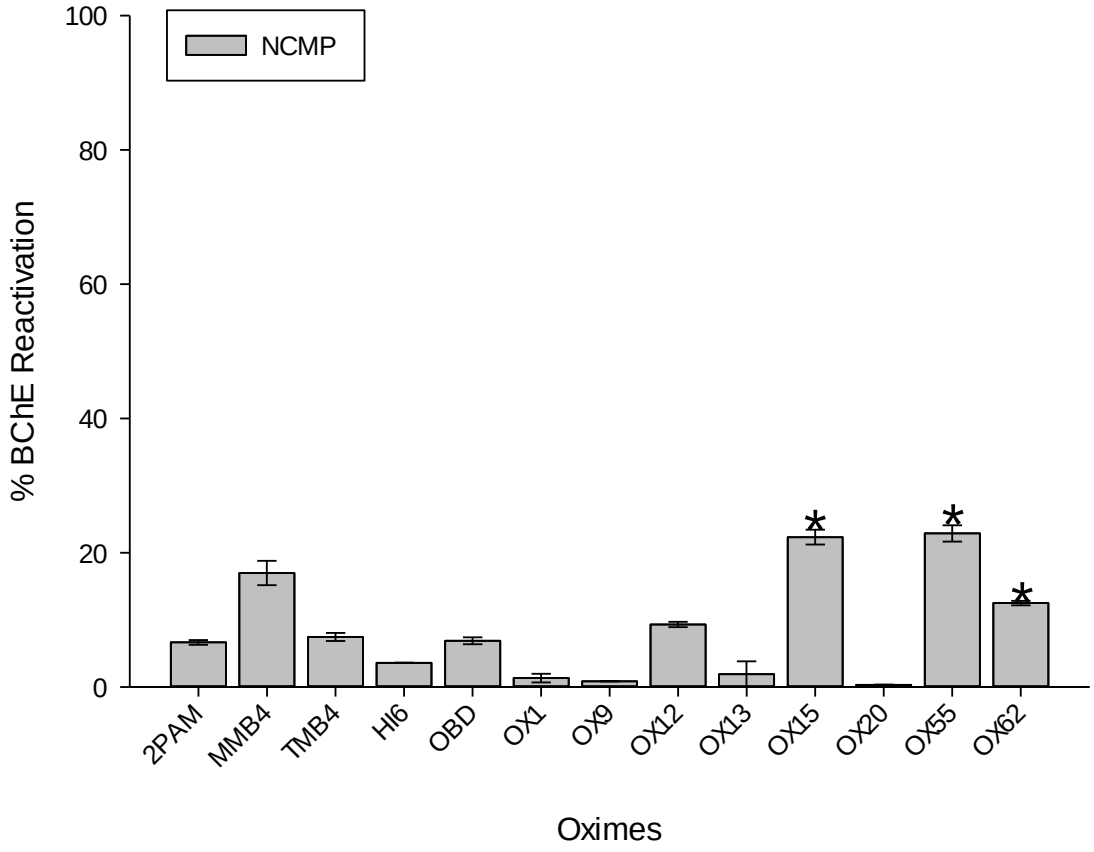



Figure 2.13 Guinea pig serum BChE reactivation after inhibition by NCMP. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 p*≤0.05 compared to 2-PAM

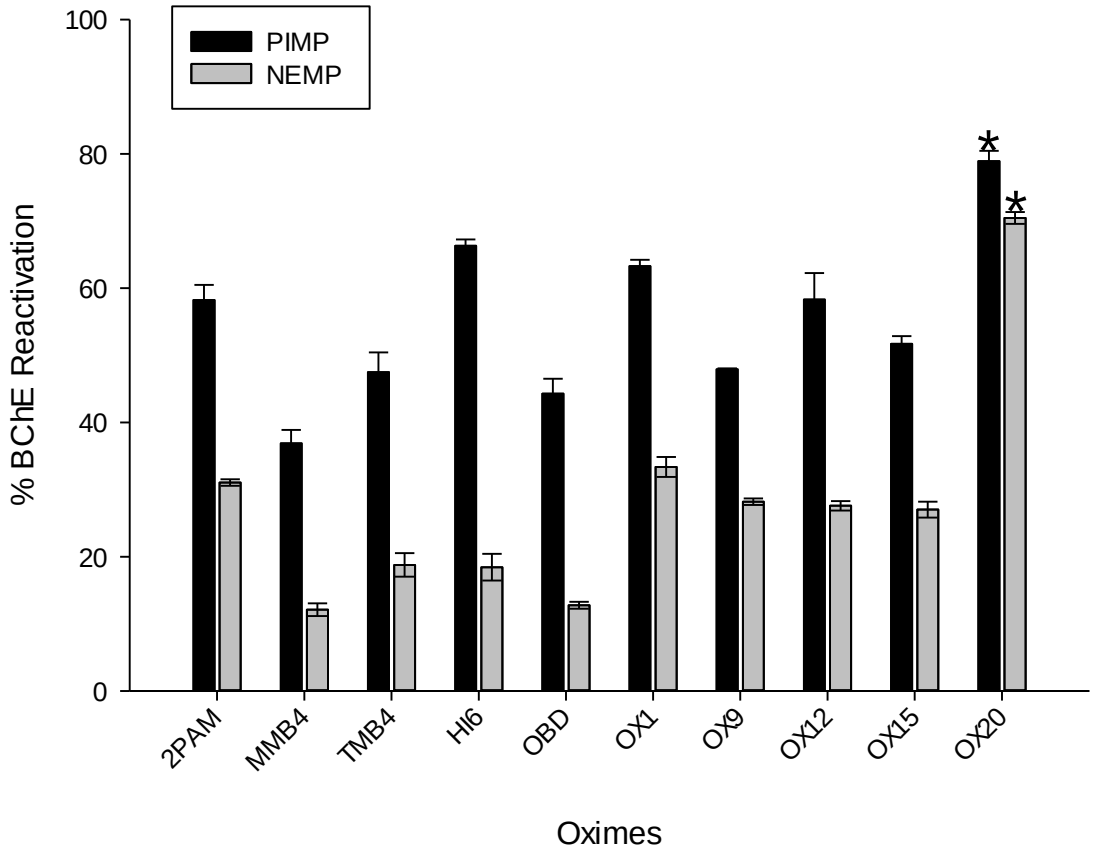



Figure 2.14 Human serum BChE reactivation after inhibition by PIMP and NEMP. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 $p^* \leq 0.05$ compared to 2-PAM

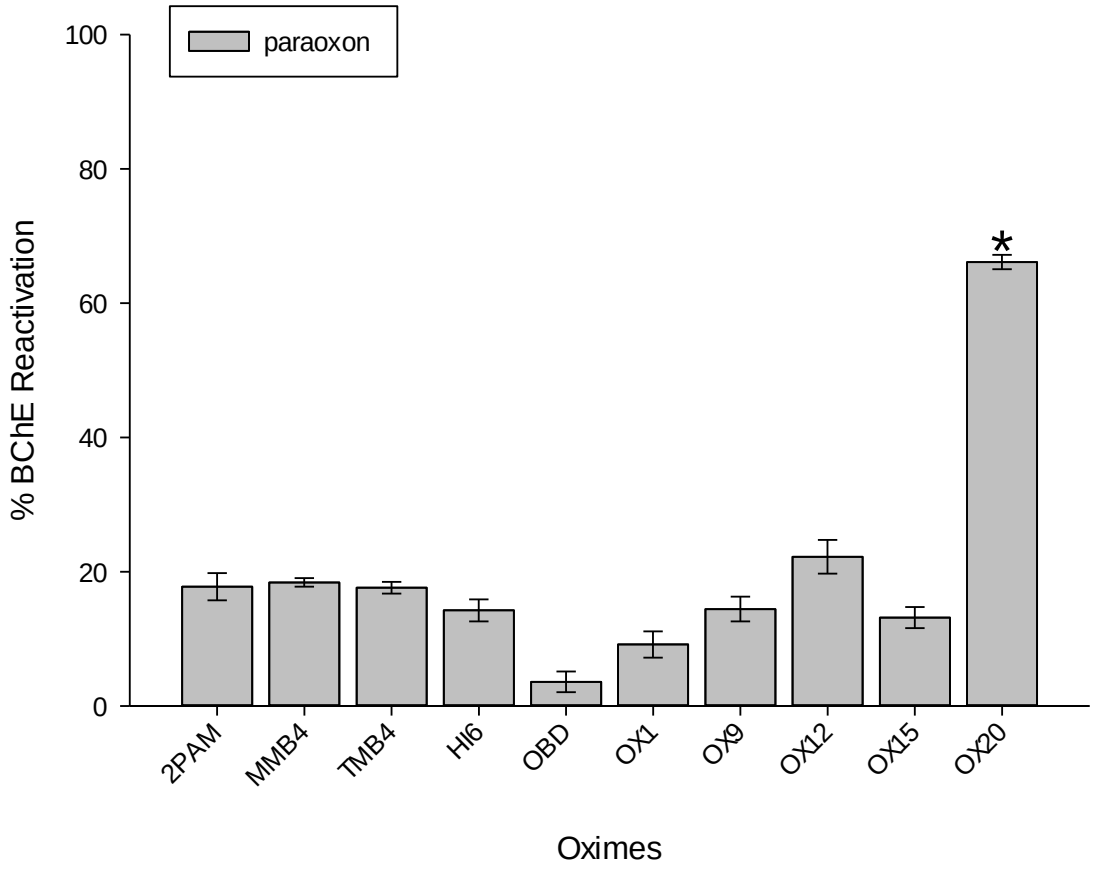



Figure 2.15 Human serum BChE reactivation after inhibition by paraoxon. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 p*≤0.05 compared to 2-PAM

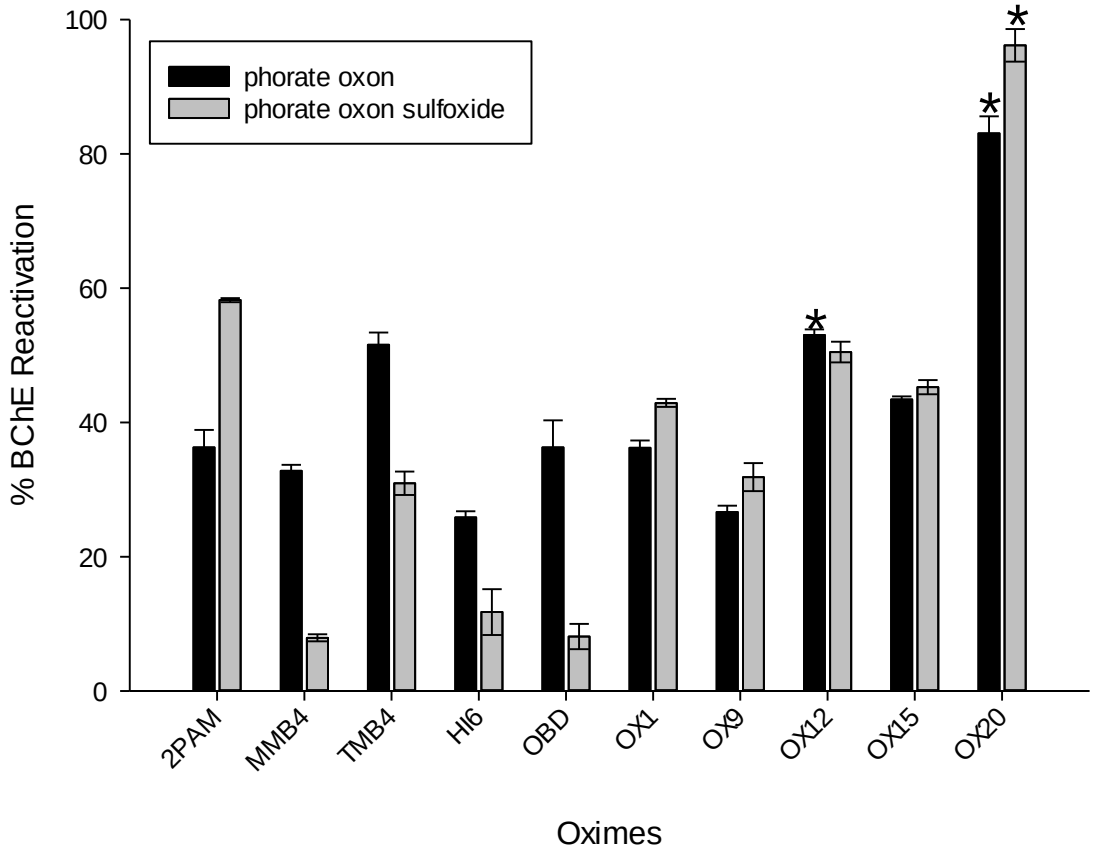



Figure 2.16 Human serum BChE reactivation after inhibition by phorate oxon and phorate oxon sulfoxide. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 p*≤0.05 compared to 2-PAM

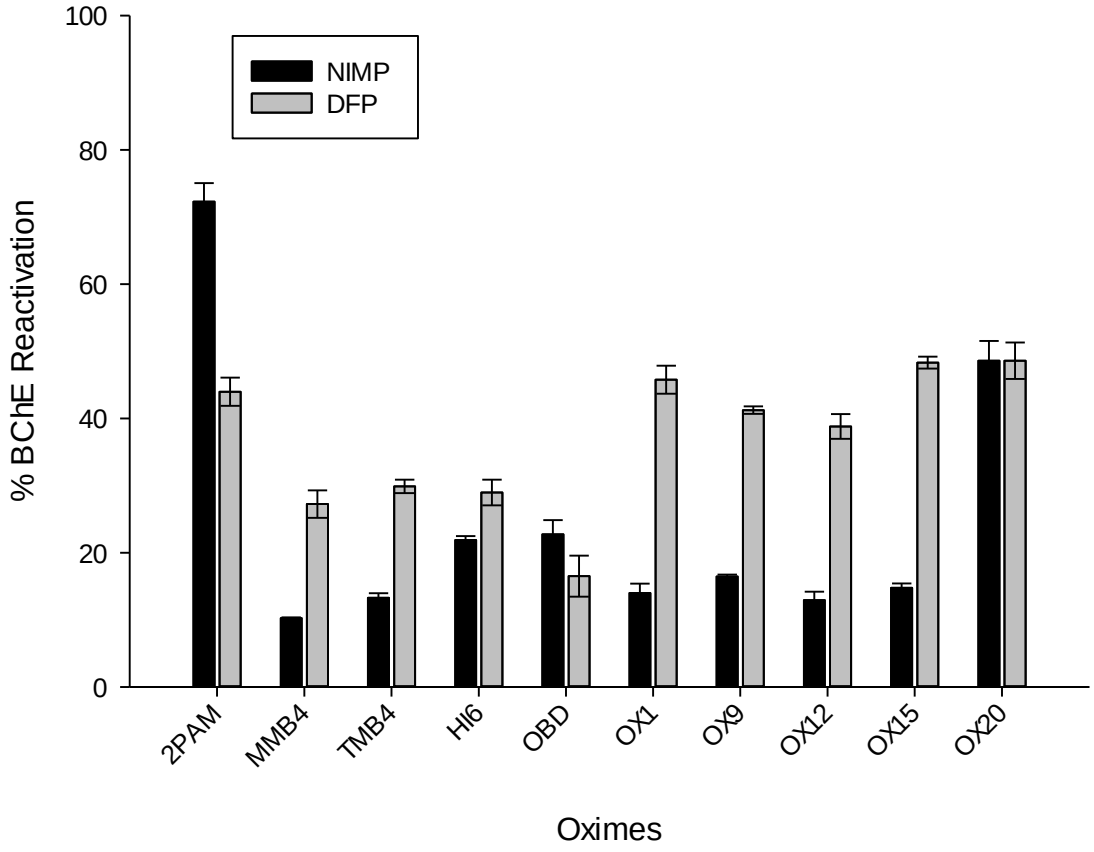



Figure 2.17 Human serum BChE reactivation after inhibition by NIMP and DFP. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3

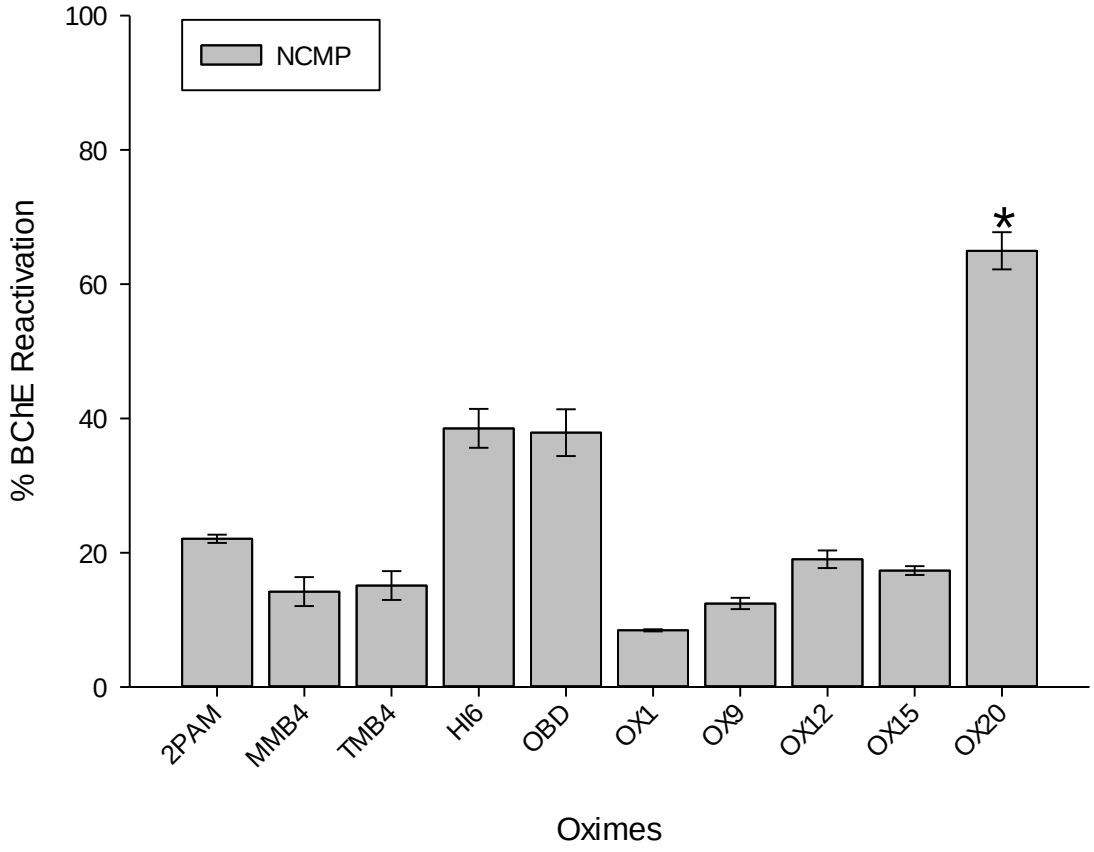



Figure 2.18 Human serum BChE reactivation after inhibition by NCMP. Data were analyzed by ANOVA and are expressed as mean ± SEM. N=3 p*≤0.05 compared to 2-PAM

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Chambers, J.E., Meek, E.C., Bennett, J.P., Bennett, S.W., Chambers, H.W., Leach, A.C…Wills, R.W. (2016). Novel substituted phenoxyalkyl pyridinium oximes enhance survival and attenuate seizure-like behavior of rats receiving lethal levels of nerve agent surrogates. *Toxicology, 339(2),* 51-57.

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# CHAPTER III

# *IN VITRO* REACTIVATION KINETICS OF RAT AND HUMAN SERUM BUTYRYLCHOLINESTERASE AND ELECTRIC EEL ACETYLCHOLINESTERASE BY NOVEL PHENOXYALKYL PYRIDINIUM OXIMES AFTER INHIBITION BY A SARINSURROGATE AND PARAOXON

## Introduction

Organophosphates (OPs) are potent inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Inhibition of AChE leads to a buildup of acetylcholine (Ach) and a persistent stimulation of the nicotinic and muscarinic cholinergic receptors throughout the central and peripheral nervous systems. A cholinergic crisis ensues resulting in increased mucosal secretions, bronchoconstriction, cardiac distress, and excitotoxic seizures. Death at lethal exposure levels happens quickly from respiratory failure if treatment is not quickly administered.

The main treatment for OPs consists of atropine sulfate and pralidoxime (2-PAM). Atropine sulfate blocks muscarinic receptor subtypes, attenuating autonomic nervous system

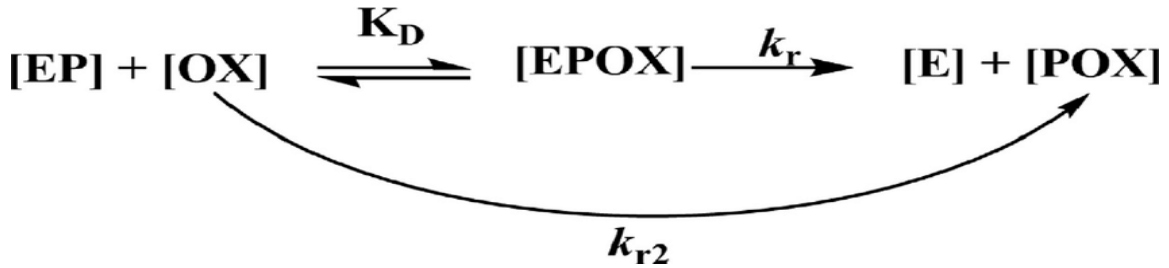
hyperactivity. Oximes such as 2-PAM are used to remove the phosphoryl group from the inhibited enzyme, thereby attenuating the over-stimulation of both the nicotinic and muscarinic receptors and allowing the body to return to homeostasis (Hobbiger and Sadler,1959). This process is called reactivation and is the primary method of oxime effectiveness. Oximes vary in their ability at reactivating OP-inhibited AChE and a broad spectrum acting reactivator has yet to be found.

In contrast to AChE, inhibition of serum and tissue BChE does not lead to toxic effects. Serum BChE serves as an endogenous scavenger of circulating toxic esters, including OPs, thus potentially reducing the amount of OP that can reach AChE and cause toxicity (Lockridge, 2015). Unfortunately the low amount of BChE in human serum (50nM) is too low to protect against a high level OP exposure (Nachon *et al.,* 2013). Administration of exogenous human BChE has afforded protection in animal models to 5.5 x $LD_{50}$ of soman and 8x $LD_{50}$ of VX (Saxena *et al.,* 2011). However, because this reaction destroys both enzyme and OP in a 1:1 stoichiometric fashion, it has been determined that the doses required to confer protection are not economically feasible (Nachon *et al.,* 2013). Research has focused on large scale production of BChE and determining if animal and recombinant sources of BChE are effective alternatives (Geyer *et al.,* 2010; Nachon *et al.,* 2002; Brazzolotto *et al.,* 2012; Li *et al.,* 2010; Huang *et al.,* 2007; Geyer *et al.,* 2010; Baldassarre *et al.,* 2009). While some promising individual results have come forth, affordable doses and effective production solutions have yet to be found.

To counter these deficiencies, using oxime reactivators to constantly regenerate OP inhibited BChE has been an emerging technique. Inhibition of BChE in the circulation followed by an oxime could potentially allow for numerous regenerations of inhibited enzyme. This could

greatly increase the number of OP molecules that could be destroyed. If effective, this could reduce the amount of enzyme needed (and thus the costs) associated with exogenous BChE treatment. Unfortunately, the current oxime platforms were originally designed to reactivate human AChE. Research suggests that current and developmental oxime platforms are not effective BChE reactivators (Aurbek *et al.*, 2009; Horn *et al.*, 2015; Radic *et al.*, 2013).

The reactivation reaction scheme of OP inhibited AChE or BChE proceeds in a two step reaction (see Scheme 1 for reactivation reaction). The oxime first approaches the inhibited enzyme and binds to it, forming a reversible OP-oxime-enzyme Michaelis-Menten type complex. The second step is the removal of the OP adduct from the enzyme (Worek *et al.*, 2013). Performing reactivation kinetics calculations produces rate constants ($K_D$, $k_r$, $k_{r2}$) that quantitatively detail the effectiveness of an oxime's ability to reactivate OP inhibited enzyme. The equilibrium constant $K_D$ represents the dissociation constant, which is inversely proportional to the affinity of the oxime towards inhibited enzyme. The first order rate constant $k_r$ details the intrinsic reactivity of the oxime, which is the ability of the oxime to remove the OP from the enzyme. The second order rate constant, $k_{r2}$, is determined by the ratio of $k_r$ and $K_D$ and determines the overall efficiency of the reactivation process (Worek *et al.*, 2007).




Scheme 3.1 Schematic representation of reactivation of OP inhibited enzyme by an oxime reactivator where [E-P] is the inhibited enzyme, [OX] is the oxime reactivator, [EPOX] is the reversible Michaelis-Menten phosphonylenzyme-oxime complex and [P-OX][E] is phosphonylated oxime and free enzyme.

The current *in vitro* investigation was designed to determine the BChE reactivation rate constants of two novel phenoxyalkyl pyridinium oximes, oxime 15 (OX15) and oxime 20 (OX20) (synthesized by the late Dr. Howard Chambers), and the currently FDA approved oxime 2-PAM in rat and human serum after inhibition by the sarin surrogate NIMP and the insecticidal metabolite paraoxon. In addition to this, the reactivation rate constants for these two oximes and 2-PAM were determined in electric eel AChE after inhibition by NIMP and paraoxon to determine if these novel oximes were better reactivators of AChE or BChE. Since Mississippi State University is not authorized to work with the actual nerve agents, our laboratories have synthesized surrogates for some of the main nerve agents (Meek *et al.*, 2012; Ohta *et al.*, 2006). These surrogates inhibit AChE and BChE with the same chemical moieties as the actual nerve agents but are safer to work with and are thus ideal for initial therapeutic studies. Because significant animal differences in oxime reactivation on AChE compared to human AChE are well documented (de Jong *et al.*, 1984; Worek *et al.*, 2002; Luo *et al.*, 2007), this study used multiple test species.

## Materials and Methods

### Materials

#### *Organophosphorus Compounds*

Nitrophenyl isopropyl methylphosphonate (NIMP; sarin surrogate) was synthesized in our laboratory (Meek *et al.*, 2012) and originally described by (Ohta *et al.*, 2006). Paraoxon (>98%) was synthesized by the late Dr. Howard Chambers. Purity was determined by Nuclear Magnetic Resonance (NMR) at Mississippi State University, Department of Chemistry.

### *Oxime Reactivators*

Two novel oximes (OX15, OX20), originally described in Chambers *et al.* (2013), were demonstrated to be the most effective BChE reactivators from chapter II and therefore progressed to chapter III for oxime reactivation kinetic determinations. 2-PAM was used as a comparison and it was purchased from Sigma-Aldrich (St. Louis, MO).

### *Tissue*

Pooled human serum and Sprague Dawley rat serum were used as sources of BChE and were purchased from Innovative Research, Novi, MI. Purified electric eel AChE was purchased from Sigma-Aldrich (St. Louis, MO). Electric eel was chosen as source of enzyme as it is an affordable and active enzyme that our laboratory has previous experience with.

## Methods

### *Determination of Butyrylcholinesterase Inhibition*

Pooled rat or human serum was diluted to appropriate assay concentrations in two 1.5ml test tubes. Rat serum was diluted to 0.250ml serum/ml and human serum was diluted to 0.025ml serum/ml in 0.05M Tris-HCl buffer, pH 7.4 (25˚C). Following this, to one tube was added 10µl of ethanol vehicle while to the other one 10µl of various concentrations of either NIMP (in EtOH) or paraoxon (in EtOH) was added. Following this, a 40 fold dilution was completed by the addition of 250µl of sample into 9.750ml of ice cold buffer to bring total volume to 10ml.

Following this, 50µl of a mixture of butyrylthiocholine (BTCh in EtOH, FC 4mM)/5,5'-dithio bis(2-nitrobenzoic acid)(DTNB, FC 1.2mM) was added to each well of a 96 well plate. The reaction was initiated by the addition of 198µl of uninhibited tissue or inhibited tissue. The uninhibited tissue samples were used to observe total enzyme activity and the inhibited tissue

samples were used to observe total inhibited enzyme activity. After these additions, the 96 well plate was immediately placed in a Biotek Plate Reader, at 37°C, where absorbance readings (412nm) were recorded every 26 seconds for 30 minutes. Linear regression was performed on the control (EtOH vehicle) and on each individual OP concentration to determine the slope. Percent inhibition of control (EtOH vehicle) was calculated for each individual OP concentration. Concentrations of OP that inhibited >90-95% were used for the reactivation experiments.

### *Determination of Butyrylcholinesterase Reactivation*

Pooled rat or human serum was diluted to appropriate assay concentrations in two 1.5ml test tubes. Rat serum was diluted to 0.250ml serum/ml and human serum was diluted to 0.025ml serum/ml in 0.05M Tris-HCl buffer, pH 7.4 (25˚C). Following this, to one tube was added 10µl of ethanol vehicle while to the other one 10µl of either NIMP (in EtOH) or paraoxon (in EtOH) was added. After 15 minutes of incubation in a shaking water bath at 37°C, samples underwent fast ultrafiltration to remove excess OP.

Briefly, for each sample (vehicle and OP sample) two aliquots of 500µl were delivered into two separate Amicon Ultra Centrifugal Filter Device tubes (Molecular Weight Cutoff 10,000 kDa) for a total of four tubes. These were placed in an Eppendorf microcentrifuge and spun at 14,000g for 5 minutes at 4°C. Proteins that were above 10,000 kDa were not filtered and were collected in the filter device tube, including the OP-BChE complex. Compounds that were below 10,000 kDa passed through the column into the filtrate collection tube, including the unbound OP. Three x five minute spins were completed. After each spin, the samples were brought to 0.5ml by the addition of 434µl buffer. After the third spin, the filter device was placed upside down into a new, clean centrifuge tube and spun for 2 minutes at 1,000g so that the

concentrated sample could be collected. The appropriate samples were combined and brought to 0.750ml by addition of 350µl buffer. Following this, a 40 fold dilution was completed by the addition of 250µl of sample into 9.750ml of ice cold buffer to bring total volume to 10ml.

Following this, 50µl of a mixture of butyrylthiocholine (BTCh in EtOH, FC 4mM)/5,5'-dithio bis(2-nitrobenzoic acid)(DTNB, FC 1.2mM) was added to each well of a 96 well plate. After this, 2µl of various concentrations of oxime (OX15, OX20, or 2-PAM) ranging from final concentrations of 1µM to 75µM were added to wells which were to contain either inhibited or uninhibited enzyme. Vehicle controls (ethanol and ethanol:DMSO) were then added (2µl) to make sure there was no vehicle induced inhibition of the enzyme. The reaction was initiated by the addition of 198µl of uninhibited tissue, inhibited tissue, or Tris HCl buffer 0.05M. The uninhibited tissue samples were used to observe total enzyme activity. The inhibited tissue samples were used to observe total inhibited enzyme activity and reactivated enzyme activity. The addition of buffer was used to observe oxime induced hydrolysis of substrate without the presence of enzyme. After these additions, the 96 well plate was immediately placed in a Biotek Plate Reader, at 37°C, where absorbance readings (412nm) were recorded every 26 seconds for 30 minutes. All conditions were performed in duplicate.

### *AChE Activity Determination*

Purified electric eel was used as source of acetylcholinesterase (AChE). Activities were monitored in a similar fashion as BChE as described previously. The only difference was that the substrate used was acetylthiocholine (ATCh in EtOH, FC 4mM). DTNB (1.2mM) was used as the chromogen in 0.05M Tris HCl buffer (pH 7.4, 25°C).

### *Inhibition of AChE by Organophosphates*

Electric eel AChE (5U) was incubated at 37°C with either ethanol/DMSO (1:1) vehicle, paraoxon or NIMP for 15 minutes in a shaking water bath to achieve >90% inhibition and was determined in a similar fashion as BChE as described previously.

### *AChE Reactivation*

Vehicle and paraoxon-inhibited or NIMP-inhibited AChE were diluted 200 fold into 8ml of 0.05M Tris HCl buffer (pH 7.4, 25°C) and 198µl were aliquoted into wells of a 96 well plate containing a mixture of 4mM ATCh, 1.2mM DTNB, and eight different oxime concentrations (ranging from FC 1µM-150µM for OX15 and 1µM-75µM for OX20 and 2-PAM) to achieve a final volume of 250µl per well and a FC enzyme activity of 0.02U. ATCh hydrolysis was continuously monitored by using a BioTek Synergy HT Multi-Mode Microplate Reader where absorbance readings (412nm) were recorded every 26 seconds for 30minutes. Sample assays were performed in duplicate. Oximes were corrected for oxime-induced hydrolysis of the substrate.

### *Data Analysis*

For wells that contained oximes, background activity resulting from oxime induced hydrolysis of the substrate without enzyme was subtracted to determine total enzyme activity. Linear regression on each individual oxime concentration's control was used to determine the slope. Non linear regression was then performed using equation 3.1 on the absorbance vs time reactivation kinetic plots (see Figure 3-1 for representative example) to determine the pseudo-first order rate constant $k_{obs}$ (Moyer *et al.*, 2018; Luo *et al.*, 2007; Worek *et al.*, 2004). Each oxime concentration that demonstrated reactivation was compared to its own oxime control for a more accurate analysis of reactivation

$$A = Yo + Vo \times t + \left(\frac{Vo}{kobs}\right)\left(e^{-kobs \times t} - 1\right) \quad (3.1)$$

Where A is absorbance, $Y_0$ is absorbance at time zero, $V_0$ is the maximum velocity (total activity of uninhibited enzyme) at time zero, t is time, and $k_{obs}$ is the pseudo first order rate constant.

Plotting the $k_{obs}$ vs oxime concentration and fitting the data to a hyperbola (Equation 3.2) allowed the calculation of the first order reactivation rate constant $k_r$, which reflects the oxime's ability to remove the OP from the inhibited enzyme and the dissociation constant $K_D$, which is inversely proportional to the oxime's affinity for the phosphorylated enzyme. The second order reactivation rate constant $k_{r2}$, which details the efficiency of the reactivation process, was calculated from equation 3.3 by taking the ratio of $k_r$ and $K_D$ (Moyer *et al.*, 2018; Luo *et al.*, 2007; Worek *et al.*, 2004).

$$k_{obs} = \frac{k_r[OX]}{K_D + [OX]} \quad (3.2)$$

$$k_{r2} = \frac{k_r}{K_D} \quad (3.3)$$

## Results

Both novel oximes proved to be efficient reactivators of NIMP- and paraoxon-inhibited electric eel AChE, indicating a high reactivity and reactivation potency (Figure 3-2-Figure 3-5). OX15 was the most effective oxime for paraoxon-inhibited AChE with a $k_{r2}$ of 3.40$mM^{-1}min^{-1}$ followed by OX20 with a $k_{r2}$ of 2.76$mM^{-1}min^{-1}$. Novel OX20 was a more effective oxime for

NIMP-inhibited AChE with a $k_{r2}$ of 2.14$mM^{-1}min^{-1}$. Both oximes were more efficient reactivators than 2-PAM at reactivating OP inhibited electric eel AChE (Table 1 and Table 2).

For paraoxon-inhibited human serum BChE, reactivation rate constants could not be determined for 2-PAM or OX15 (Table 3). This was because these oximes displayed slow rates of reactivation. When oximolysis was subtracted from these values, a negative number was produced which prevented the calculation of the rate constants. OX20 indicated a modest reactivity towards paraoxon-inhibited human serum BChE with a $k_{r2}$ of 0.593$mM^{-1}min^{-1}$ (Table 3). For NIMP-inhibited human serum BChE, novel OX20 indicated impressive reactivation efficiency with a $k_{r2}$ of 9.78$mM^{-1}min^{-1}$ followed by oxime 15, with a $k_{r2}$ 4.36$mM^{-1}min^{-1}$. Both oximes were more effective reactivators of NIMP-inhibited human serum BChE than 2-PAM (Table 4).

For OP-inhibited rat BChE, all oximes displayed low intrinsic reactivation potency ($k_r$) and high affinity for the inhibited enzyme ($K_D$) which produced higher $k_{r2}$ values (Table 5 and Table 6). Novel OX15 proved to be the most effective reactivator of paraoxon-inhibited rat BChE with a second order rate constant of 142.73$mM^{-1}min^{-1}$ while OX20 was the most effective reactivator for NIMP-inhibited rat serum BChE with a second order rate constant of 1.02$mM^{-1}min^{-1}$. Rate constants for novel OX15 could not be determined for NIMP-inhibited rat serum BChE but OX15 was an effective reactivator of paraoxon-inhibited rat BChE, with a second order rate constant of 21.15$mM^{-1}min^{-1}$. Both novel oximes were more effective reactivators than 2-PAM at reactivating OP inhibited rat BChE.

## Discussion

Reactivation rate constants for OP inhibited electric eel AChE and rat and human serum BChE were determined for two novel phenoxyalkyl pyridinium oximes, OX15 and OX20, and 2-

PAM. Tested OPs consisted of the insecticidal metabolite paraoxon and the sarin surrogate NIMP. Results indicated high intrinsic oxime reactivity for NIMP- and paraoxon-inhibited electric eel AChE for OX15 and OX20. Both oximes displayed similar reactivity with paraoxon-inhibited electric eel AChE, with a $k_r$ of $0.2min^{-1}$ for both oximes, and NIMP-inhibited electric eel AChE with a $k_r$ of $0.17min^{-1}$. 2-PAM also showed high reactivity with paraoxon-inhibited electric eel AChE with a $k_r$ of $0.19min^{-1}$, but was not better than OX15 or OX20. 2-PAM displayed poor reactivity towards NIMP-inhibited electric eel AChE. Dissociation constants, reflected by $K_D$, for the two novel oximes were less than for 2-PAM indicating higher affinity and reactivity towards paraoxon- and NIMP-inhibited enzyme. These values were around 100µM for both OP compounds and 2-PAM. The second order rate constants for 2-PAM for NIMP (sarin surrogate) and paraoxon were close to that of the second order rate constants of sarin and paraoxon inhibited human AChE (Worek *et al.*, 2002; Worek *et al.*, 2004; Worek *et al.*, 2011), indicating that electric eel AChE is a good model for human AChE.

For rat and human BChE rate constants, OX20 was the only oxime that showed sufficient reactivity towards NIMP-inhibited human serum BChE, with a $k_r$ of $0.1min^{-1}$ and an extremely high affinity towards the NIMP-inhibited enzyme (less than 20µM). All oximes displayed high affinity (less than 10µM) but low reactivity ($k_r$ of less than $0.015min^{-1}$) towards paraoxon-inhibited rat BChE.

According to Worek *et al.* (2011), a minimum requirement for an effective oxime reactivator is a $k_r$ of $>0.1min^{-1}$ and a $K_D$ of 100µM or lower. Both novel oximes met these requirements for paraoxon- and NIMP-inhibited electric eel AChE while OX20 also met these requirements for NIMP-inhibited human serum BChE, indicating strong reactivity towards structurally different OPs and different enzymes. The second order rate constant, $k_{r2}$, which

details the overall efficiency of the reactivation process, was higher for our novel oximes than 2-PAM for all compounds and enzymes tested.

The oximes displayed lower intrinsic reactivity ($k_r$) towards OP-inhibited BChE than for OP-inhibited AChE. This could be due to the fact that the larger active site in BChE compared to AChE could prevent proper orientation of the oxime towards the phosphylated product (Vellom *et al.,* 1993). However, this poor reactivity was offset by a very high binding affinity, especially for OP-inhibited rat BChE. Saturation was reached very quickly with the oximes tested for OP-inhibited rat BChE, which could be explained by the very high binding affinity. The smaller active site in rat BChE compared to human BChE has been noted (Boeck *et al.*, 2002), and this could be an important determinant of oxime binding for these small, lipophilic, novel oximes.

## Conclusion

The results indicate that our oximes are more effective reactivators of electric eel AChE and human and rat serum BChE for paraoxon- and NIMP-inhibited enzyme than the current oxime therapeutic 2-PAM. These novel phenoxyalkyl pyridinium oximes have added lipophilic moieties and were synthesized to improve the amount of oxime that can enter into brain and protect against OP-induced brain damage. Indeed, data from our lab has shown increased survival rates and neural protection in rats after exposure to a sarin surrogate and paraoxon when compared to 2-PAM (Chambers *et al.,* 2016; Dail *et al.,* 2018). A possible reasoning into this increased survivability and brain protection could be an increase in the detoxication of OP molecules in the circulation by oxime assisted reactivation of OP-inhibited serum BChE. Oxime induced reactivation of OP inhibited BChE would theoretically allow fewer OP molecules to reach critical AChE target areas such as the brain. Additionally, based on the electric eel AChE data, these oximes do possess the capability to effectively reactivate AChE. Reactivation of

AChE and BChE could provide a dual therapeutic effect. Perhaps not too surprisingly, OX20 has emerged as one of our lead *in vivo* oximes. Data represented here has shown that OX20 is an effective OP-inhibited AChE and BChE reactivator of a sarin surrogate and paraoxon.

Additional studies into OP inhibited human AChE and more structurally diverse OPs are needed to assess the true effectiveness of these novel oximes. A critical need exists to develop oximes that are broad spectrum and neuroprotective and these data suggest that our novel phenoxyalkyl pyridinium oximes are showing promise as broad spectrum acting and neural protecting oxime reactivators.

Table 3.1 Reactivation rate constants $k_r$ and $k_{r2}$, and $K_D$ for 2-PAM and novel oximes 15 and 20 for paraoxon inhibited electric eel AChE.

| Oxime | $K_D$(μM) | $k_r$ ($min^{-1}$) | $k_{r2}$ ($mM^{-1}min^{-1}$) |
|---|---|---|---|
| 2-PAM | 95±16 | 0.194±0.038 | 2.09±0.76 |
| Oxime 15 | 70±59 | 0.222±0.023 | 3.40±0.81 |
| Oxime 20 | 84±34 | 0.220±0.163 | 2.76±0.81 |

Data are represented as mean± SD (n=2 observations).

Table 3.2 Reactivation rate constants $k_r$ and $k_{r2}$, and $K_D$ for 2-PAM and novel oximes 15 and 20 for NIMP inhibited electric eel AChE.

| Oxime | $K_D$(μM) | $k_r$ ($min^{-1}$) | $k_{r2}$ ($mM^{-1}min^{-1}$) |
|---|---|---|---|
| 2-PAM | 126±148 | 0.051±0.013 | 1.09±1.16 |
| Oxime 15 | 108±34 | 0.171±0.011 | 1.63±0.40 |
| Oxime 20 | 79±26 | 0.170±0.054 | 2.14±0.04 |

Data are represented as mean± SD (n=2 observations).

Table 3.3 Reactivation rate constants $k_r$, $k_{r2}$, and $K_D$ for 2-PAM and novel oximes 15 and 20 for paraoxon inhibited human serum BChE.

| Oxime | $K_D$(μM) | $k_r$ ($min^{-1}$) | $k_{r2}$ ($mM^{-1}min^{-1}$) |
|---|---|---|---|
| 2-PAM | Ø | Ø | Ø |
| Oxime 15 | Ø | Ø | Ø |
| Oxime 20 | 84±20 | 0.048±0.012 | 0.59±0.05 |

Data are represented as mean± SD (n=2-3 observations).

Table 3.4 Reactivation rate constants $k_r$, $k_{r2}$, and $K_D$ for 2-PAM and novel oximes 15 and 20 for NIMP inhibited human serum BChE.

| Oxime | $K_D$(μM) | $k_r$ ($min^{-1}$) | $k_{r2}$ ($mM^{-1}min^{-1}$) |
|---|---|---|---|
| 2-PAM | 13±15 | 0.008±0.002 | 1.23±1.21 |
| Oxime 15 | 60±58 | 0.078±0.029 | 4.36±6.17 |
| Oxime 20 | 18±18 | 0.100±0.012 | 9.78±3.34 |

Data are represented as mean± SD (n=2-3 observations).

Table 3.5 Reactivation rate constants $k_r$, $k_{r2}$, and $K_D$ for 2-PAM and novel oximes 15 and 20 for paraoxon inhibited rat serum BChE.

| Oxime | $K_D$(μM) | $k_r$ ($min^{-1}$) | $k_{r2}$ ($mM^{-1}min^{-1}$) |
|---|---|---|---|
| 2-PAM | 8.14±11 | 0.010±0.001 | 58.51±81.80 |
| Oxime 15 | 0.52±0.67 | 0.011±0.005 | 142.73±191 |
| Oxime 20 | 0.42±0.22 | 0.012±0.008 | 38.74±39.97 |

Data are represented as mean± SD (n=2-3 observations).

Table 3.6 Reactivation rate constants $k_r$ and $k_{r2}$, and $K_D$ for 2-PAM and novel oximes 15 and 20 for NIMP inhibited rat serum BChE.

| Oxime | $K_D$(μM) | $k_r$ ($min^{-1}$) | $k_{r2}$ ($mM^{-1}min^{-1}$) |
|---|---|---|---|
| 2-PAM | 278±295 | 0.060±0.058 | 0.24±0.04 |
| Oxime 15 | Ø | Ø | Ø |
| Oxime 20 | 24±8 | 0.024±0.005 | 1.02±0.29 |

Data are represented as mean± SD (n=2-3 observations).

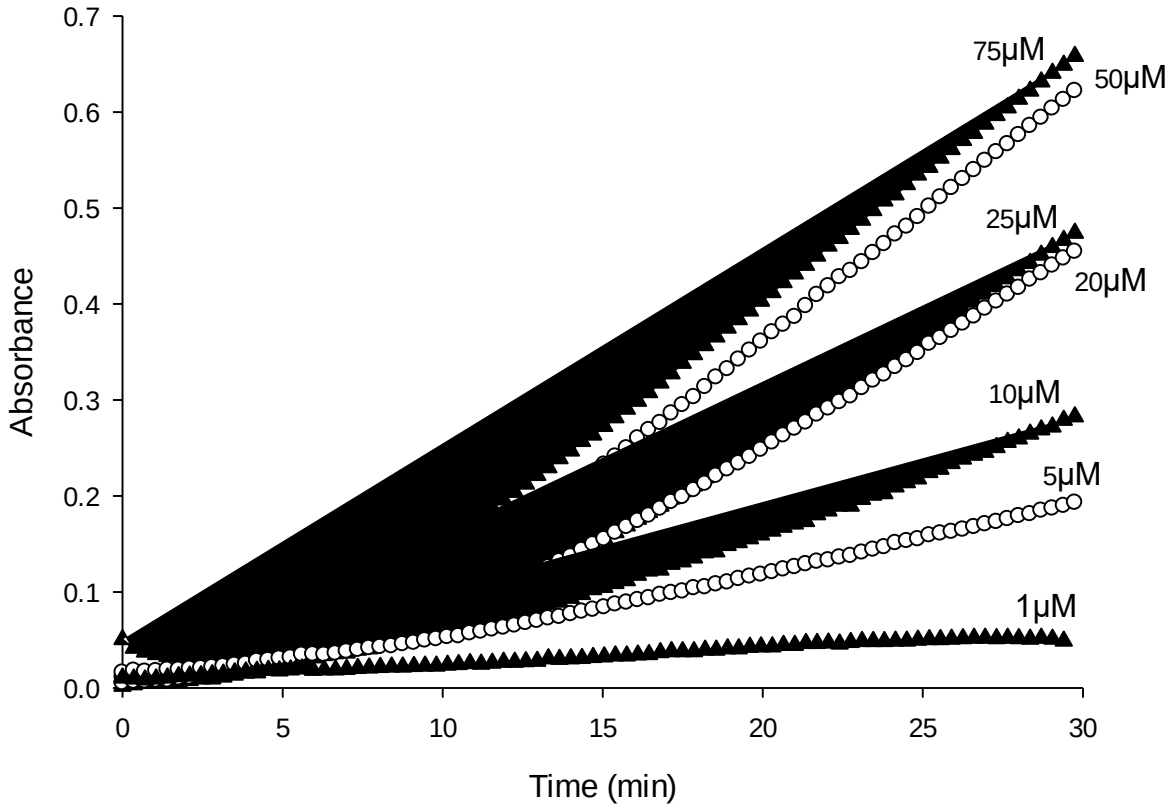



Figure 3.1 Representative absorbance vs time plot of the reactivation kinetics of NIMP inhibited electric eel AChE by OX20 (1μM-75μM).

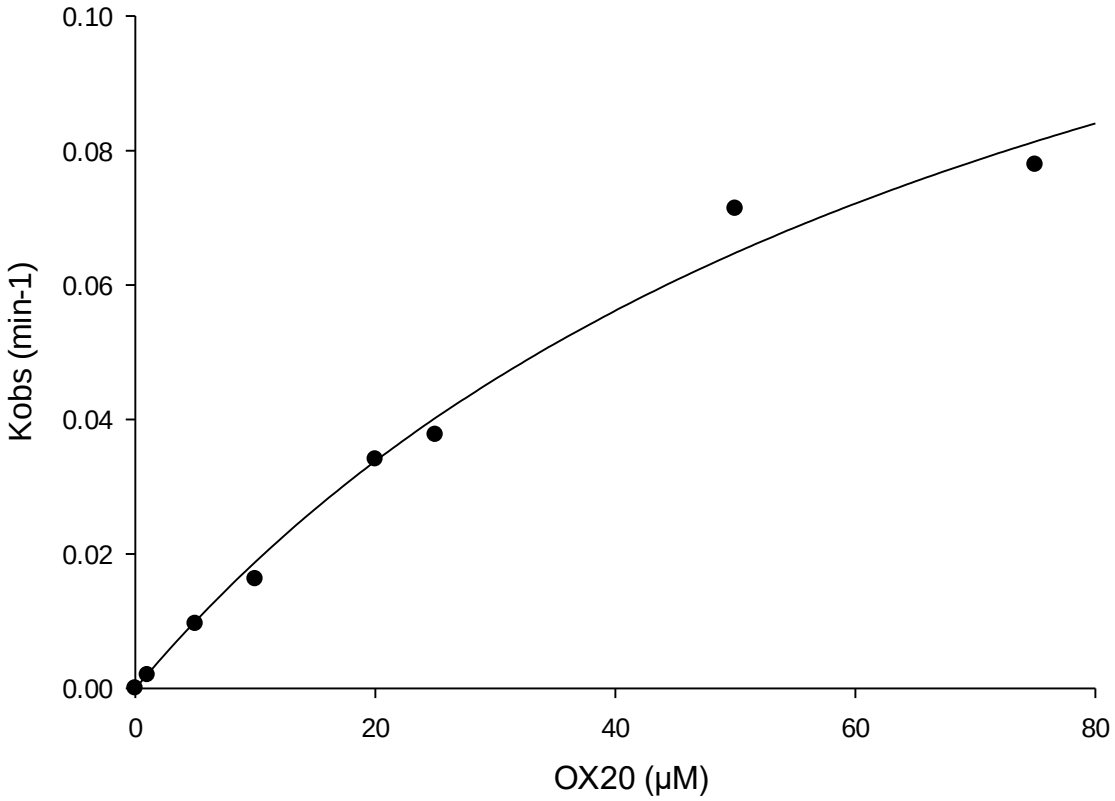



Figure 3.2 Plot of $k_{obs}$ for NIMP inhibited electric eel AChE reactivated by various concentrations of OX20. $k_{obs}$ vs [OX20] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.98

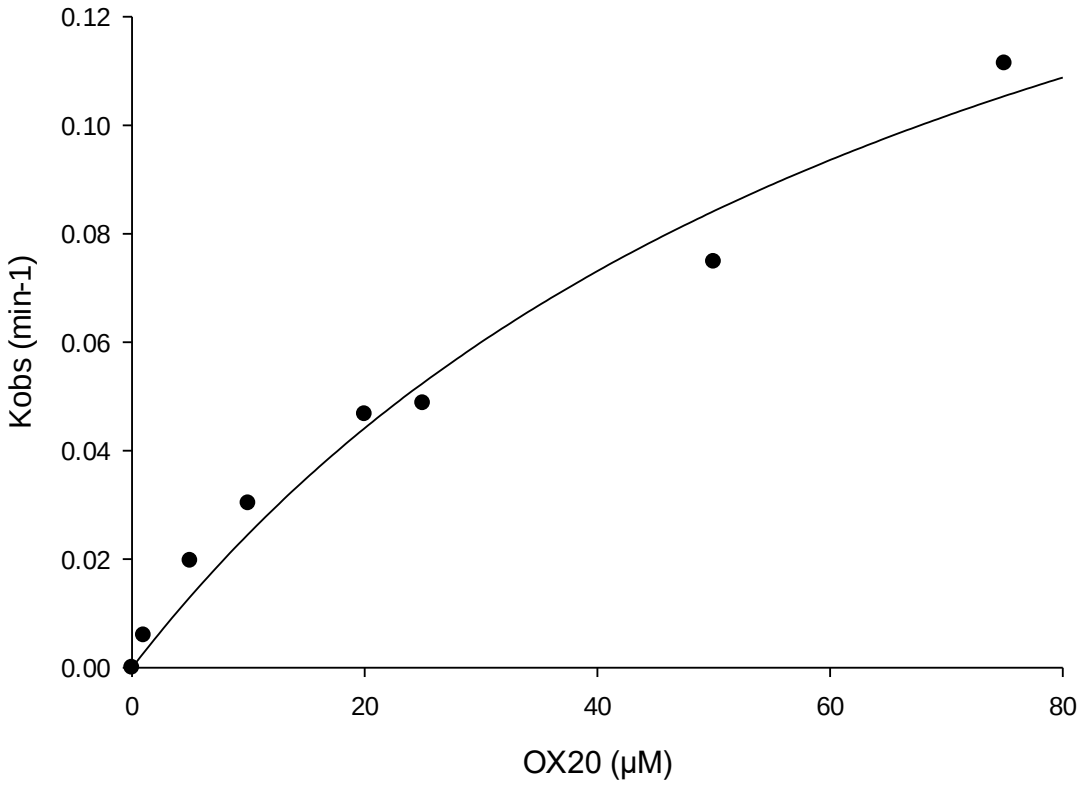



Figure 3.3 Plot of $k_{obs}$ for paraoxon inhibited electric eel AChE reactivated by various concentrations of OX20. $k_{obs}$ vs [OX20] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.97

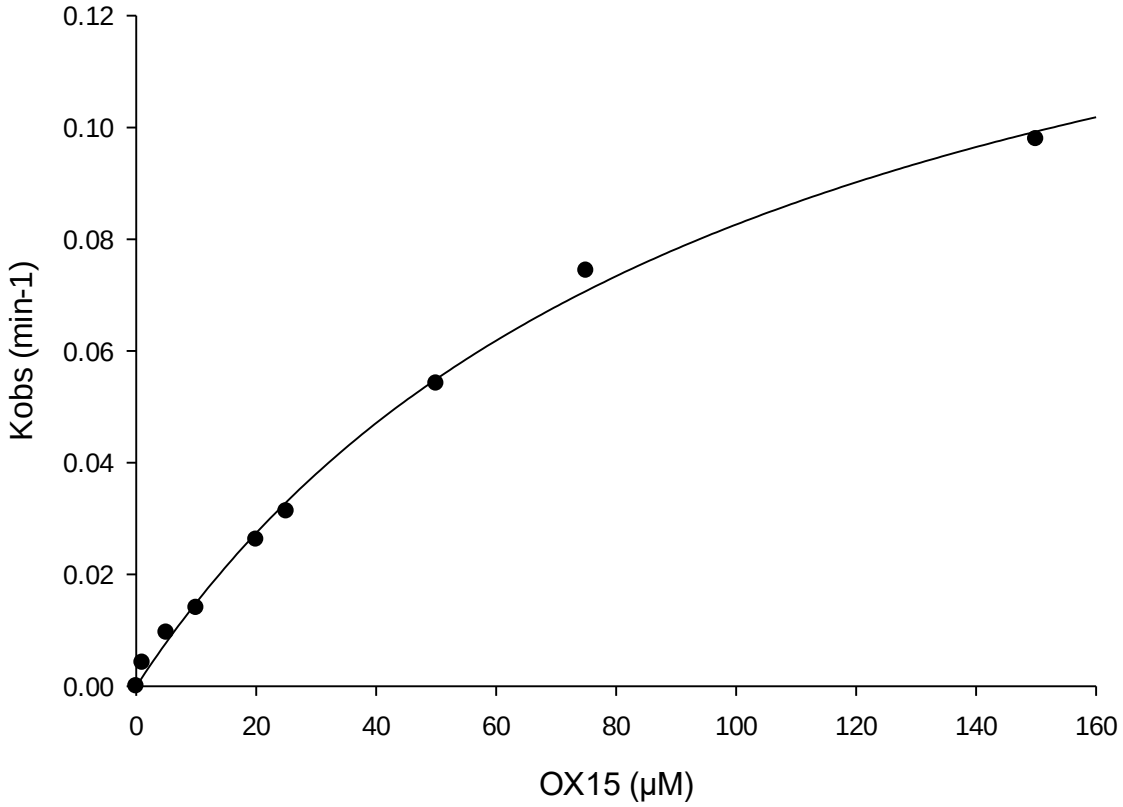



Figure 3.4 Plot of $k_{obs}$ for NIMP inhibited electric eel AChE reactivated by various concentrations of OX15. $k_{obs}$ vs [OX15] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.99

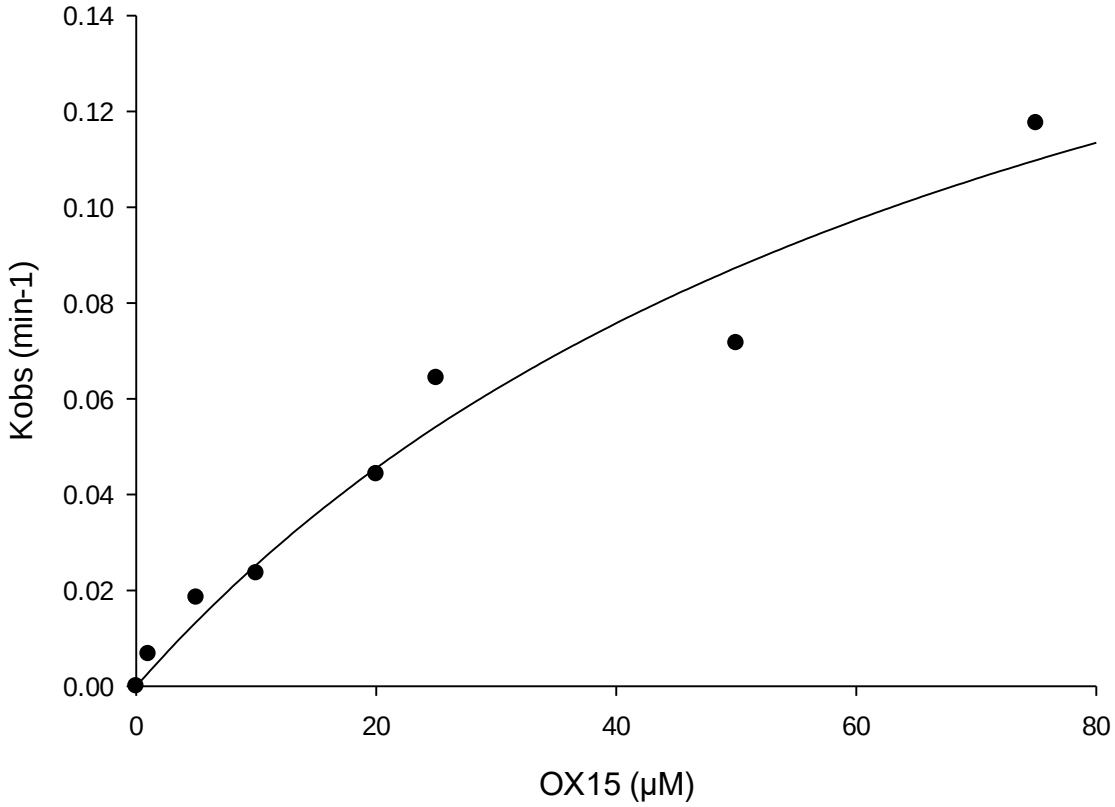



Figure 3.5 Plot of $k_{obs}$ for paraoxon inhibited electric eel AChE reactivated by various concentrations of OX15. $k_{obs}$ vs [OX15] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.95

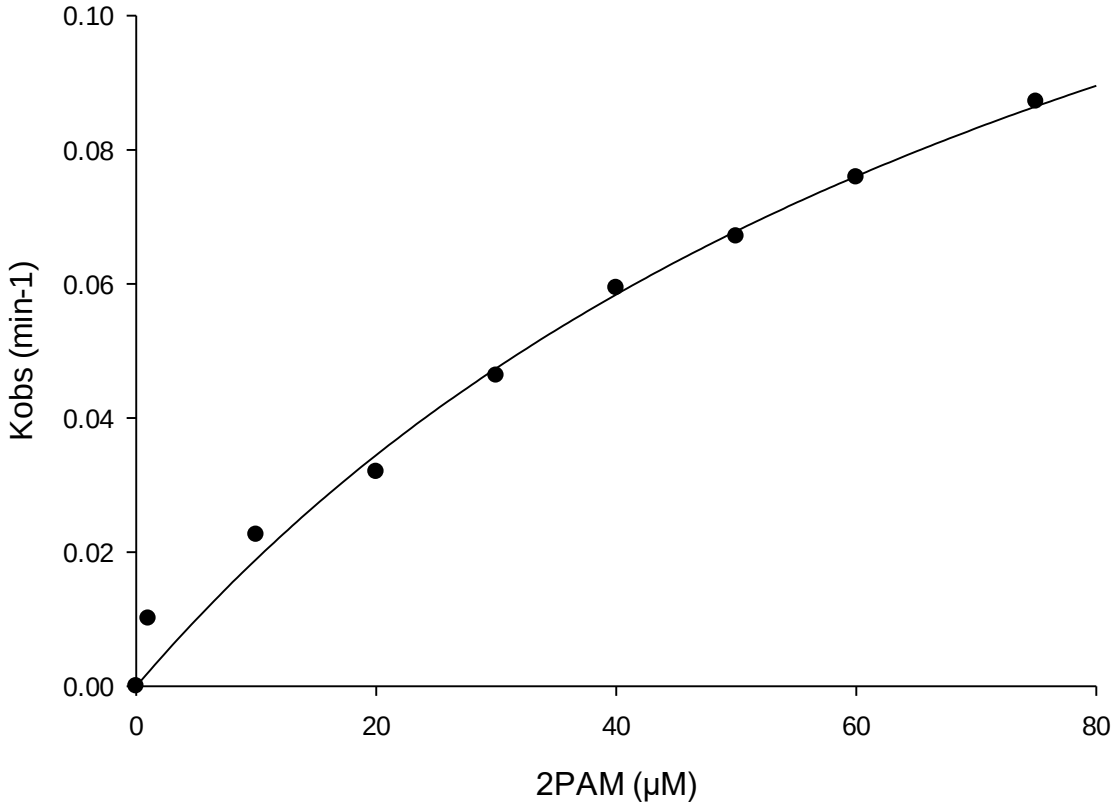



Figure 3.6 Plot of kobs for NIMP inhibited electric eel AChE reactivated by various concentrations of 2PAM. kobs vs [2PAM] allowed the determination of the rate constant kr and affinity constant KD. R2 of 0.91

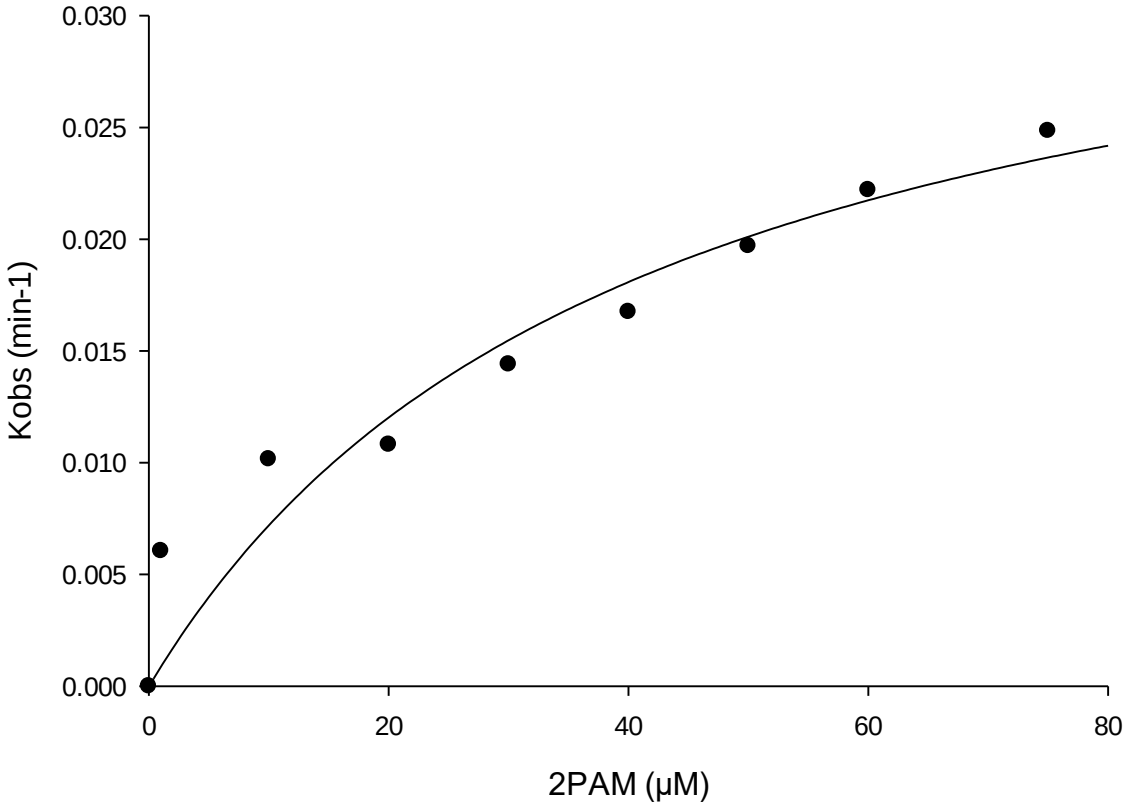



Figure 3.7 Plot of $k_{obs}$ for paraoxon inhibited electric eel AChE reactivated by various concentrations of 2PAM. $k_{obs}$ vs [2PAM] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.98

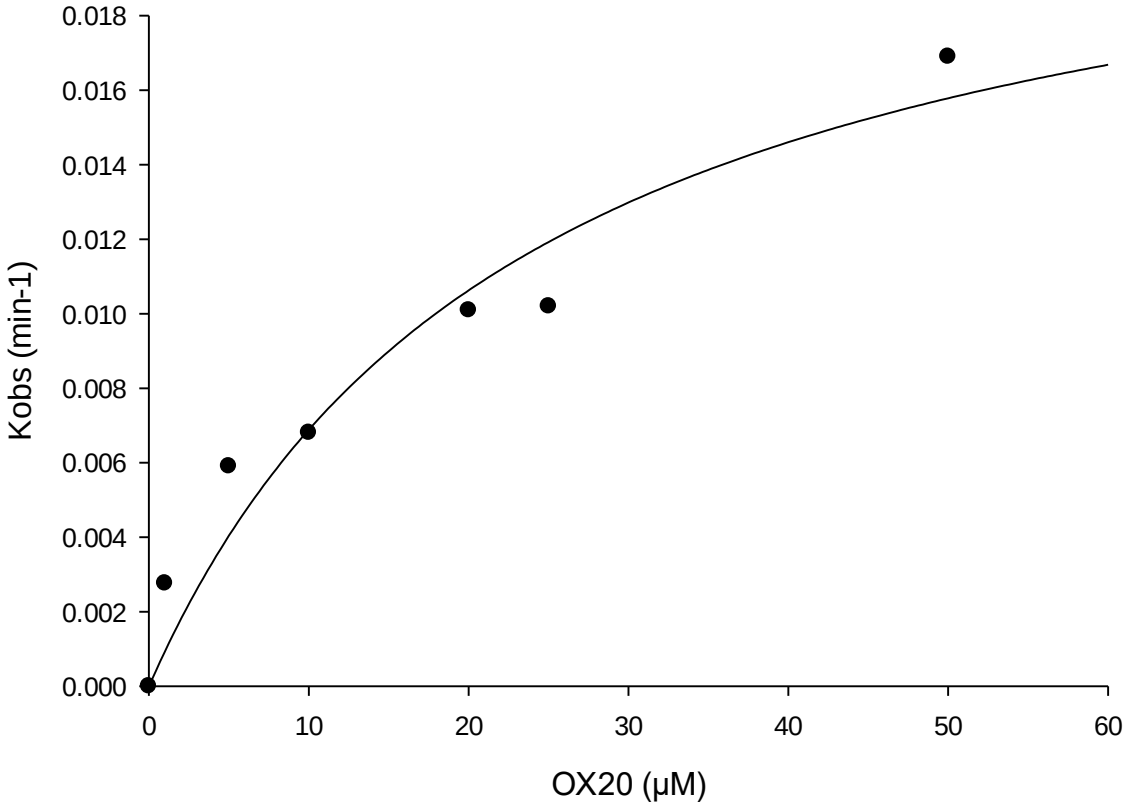



Figure 3.8 Plot of $k_{obs}$ for NIMP inhibited rat serum BChE reactivated by various concentrations of OX20. $k_{obs}$ vs [OX20] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.93

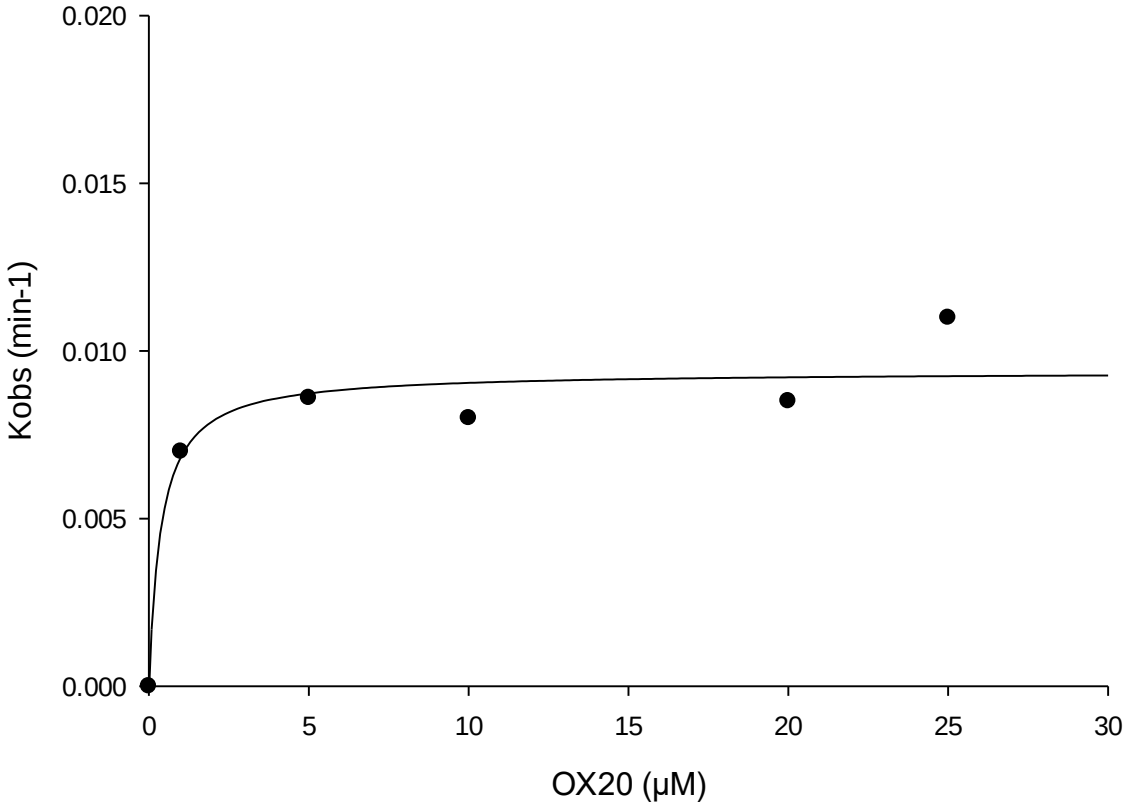



Figure 3.9 Plot of $k_{obs}$ for paraoxon inhibited rat serum BChE reactivated by various concentrations of OX20. $k_{obs}$ vs [OX20] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.93

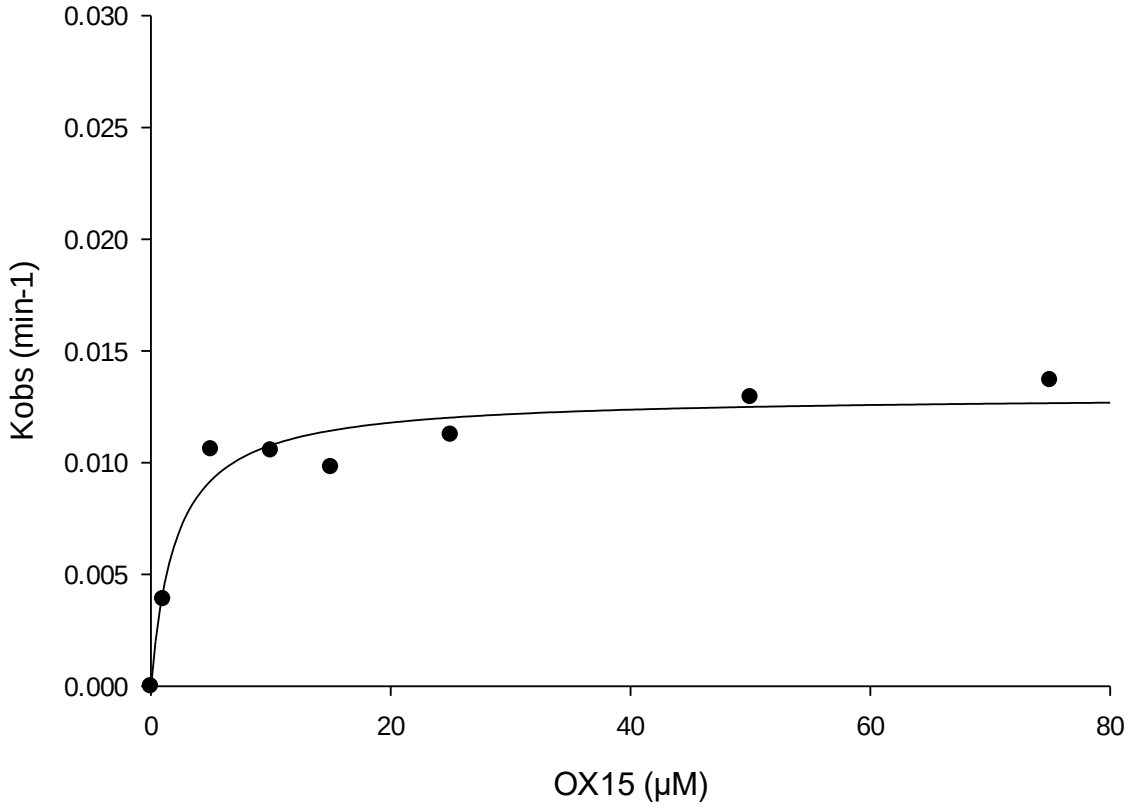



Figure 3.10 Plot of $k_{obs}$ for paraoxon inhibited rat serum BChE reactivated by various concentrations of OX15. $k_{obs}$ vs [OX15] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.95

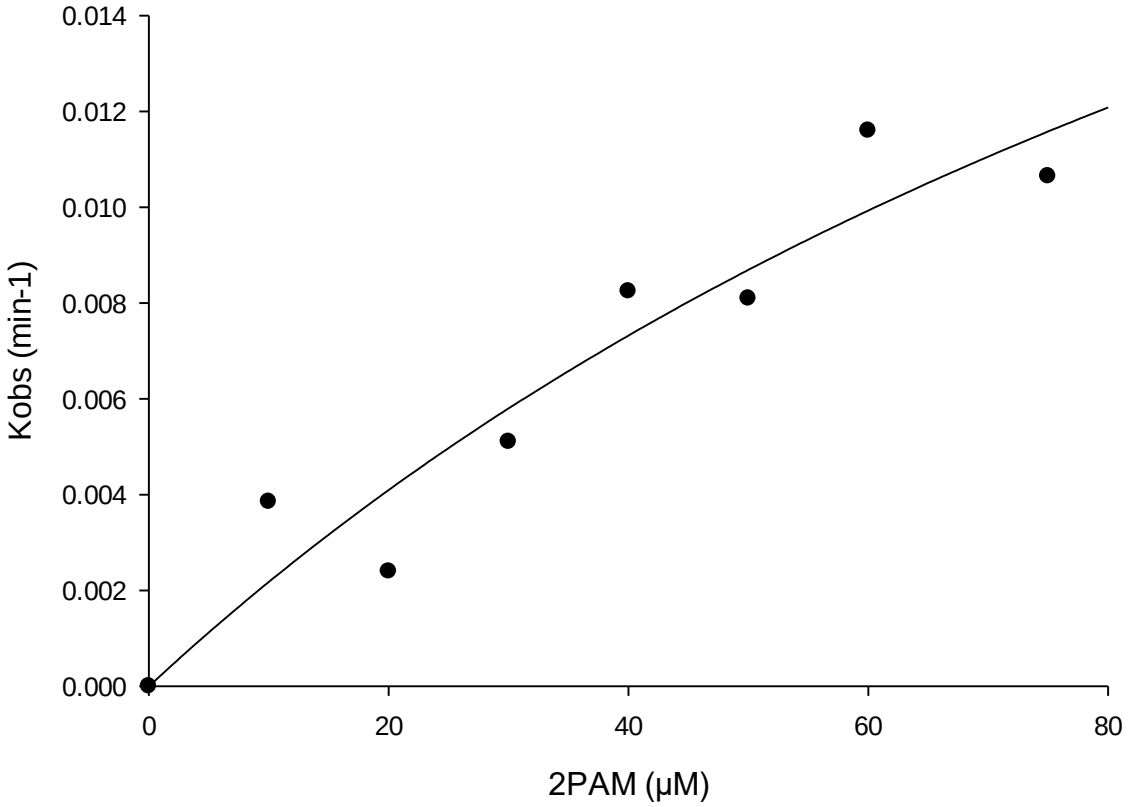



Figure 3.11 Plot of $k_{obs}$ for NIMP inhibited rat serum BChE reactivated by various concentrations of 2PAM. $k_{obs}$ vs [2PAM] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.90

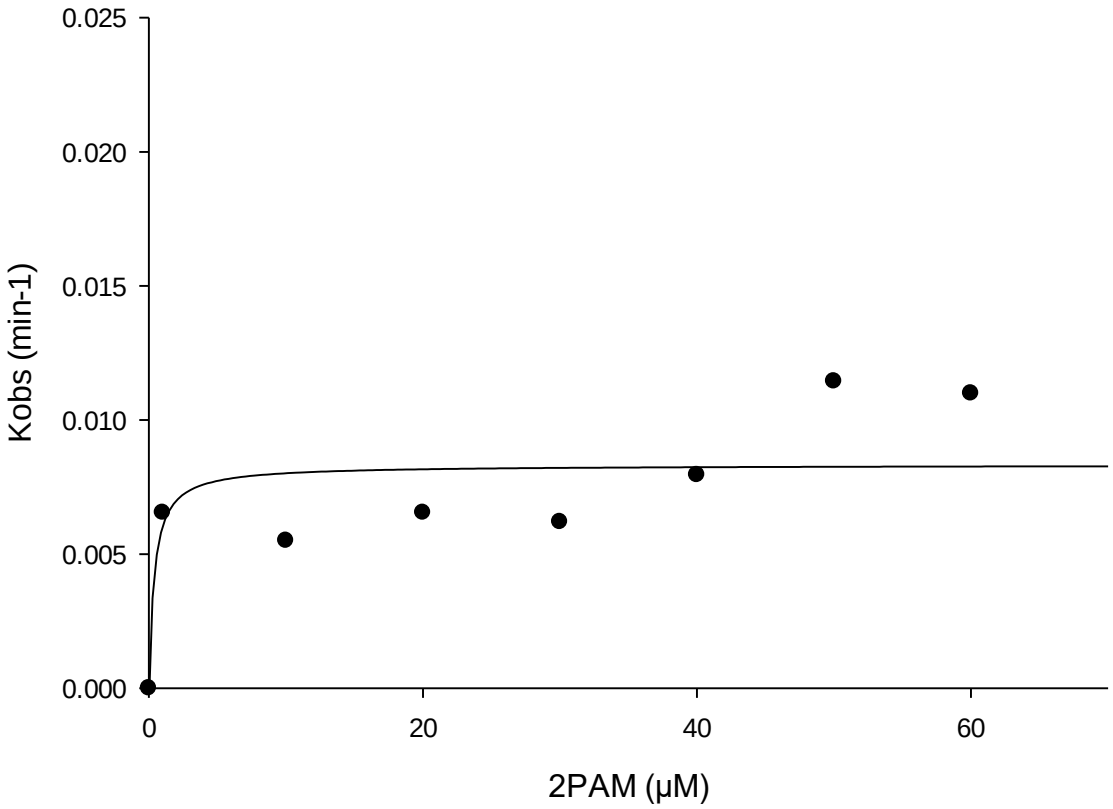



Figure 3.12 Plot of $k_{obs}$ for paraoxon inhibited rat serum BChE reactivated by various concentrations of 2PAM. $k_{obs}$ vs [2PAM] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.65

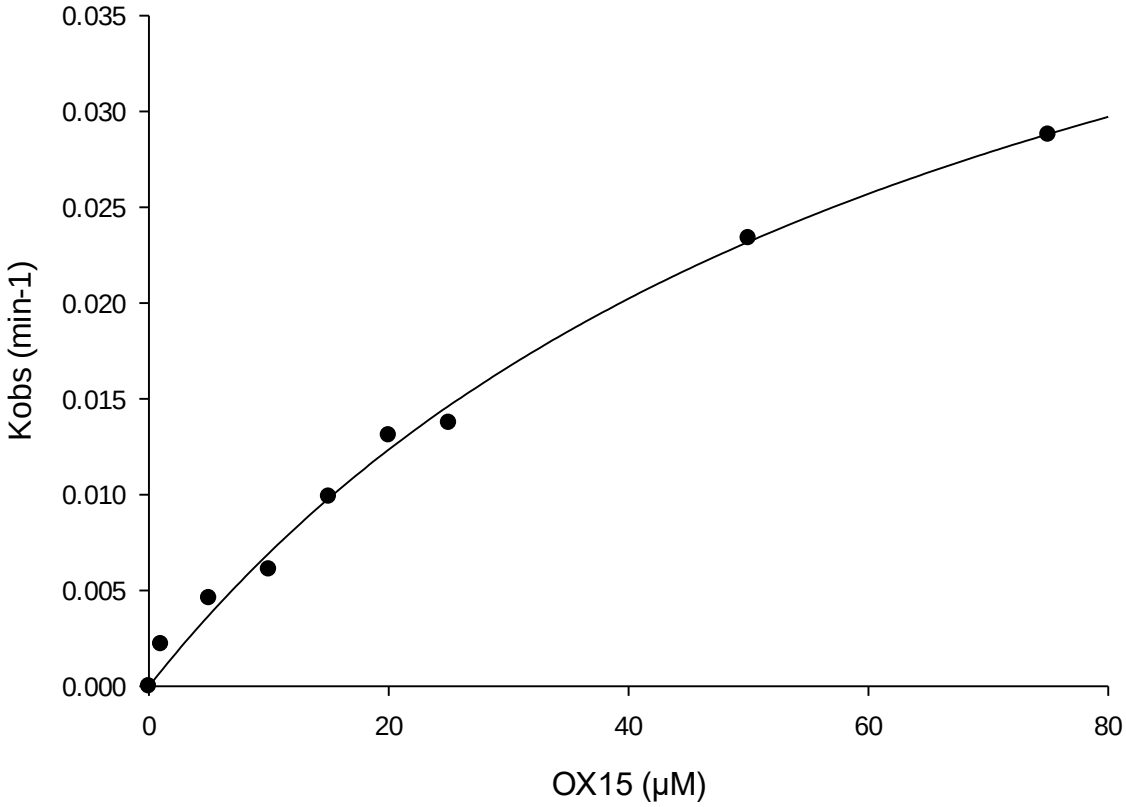



Figure 3.13 Plot of $k_{obs}$ for NIMP inhibited human serum BChE reactivated by various concentrations of OX15. $k_{obs}$ vs [OX15] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.99

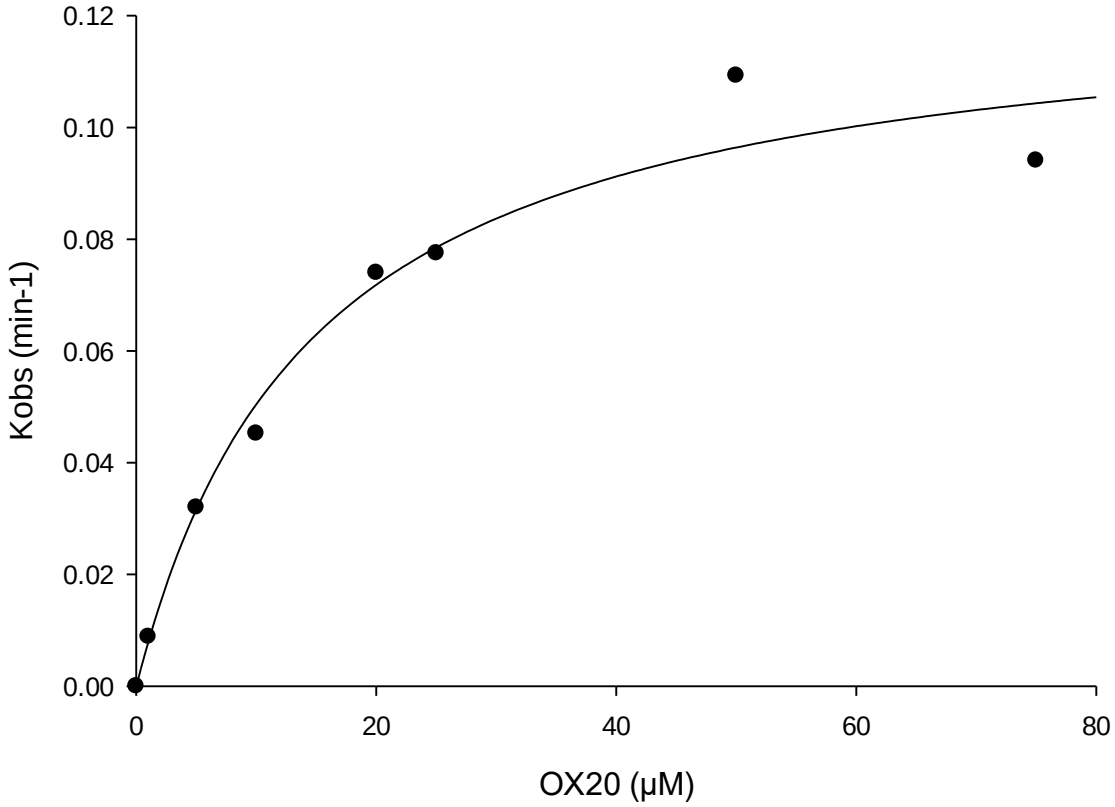



Figure 3.14 Plot of $k_{obs}$ for NIMP inhibited human serum BChE reactivated by various concentrations of OX20. $k_{obs}$ vs [OX20] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.97

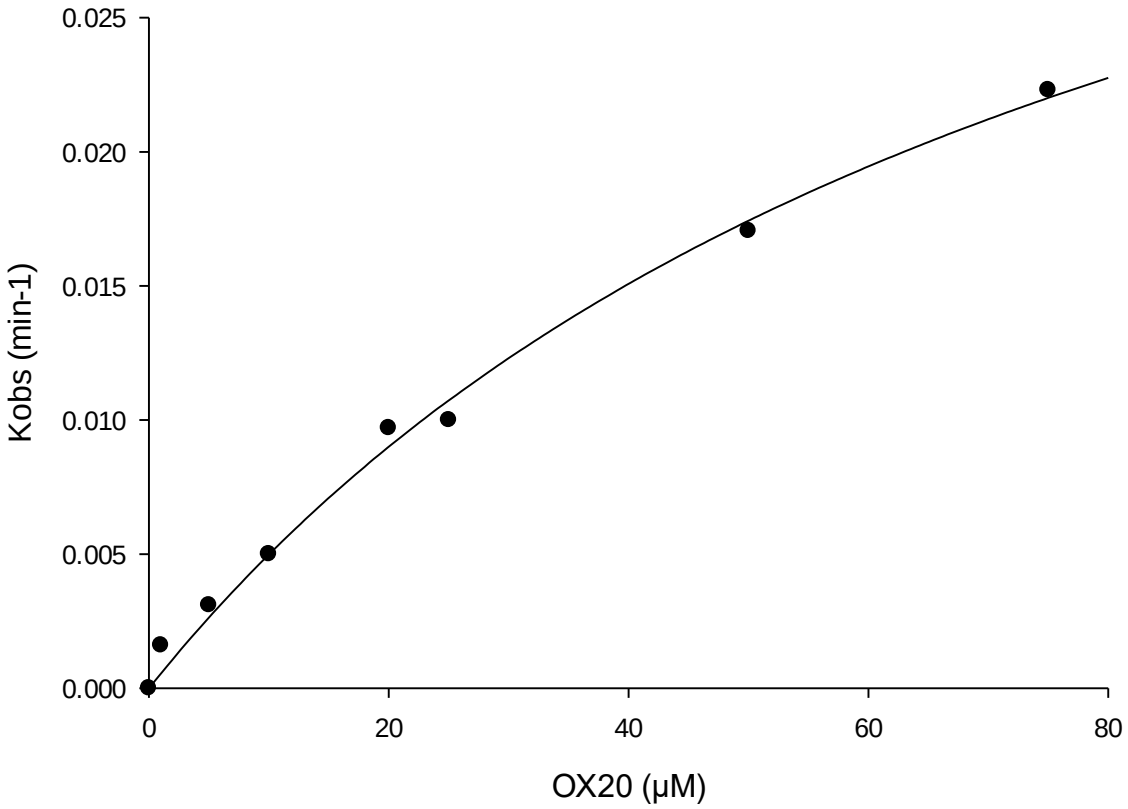



Figure 3.15 Plot of $k_{obs}$ for paraoxon inhibited human serum BChE reactivated by various concentrations of OX20. $k_{obs}$ vs [OX20] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.99

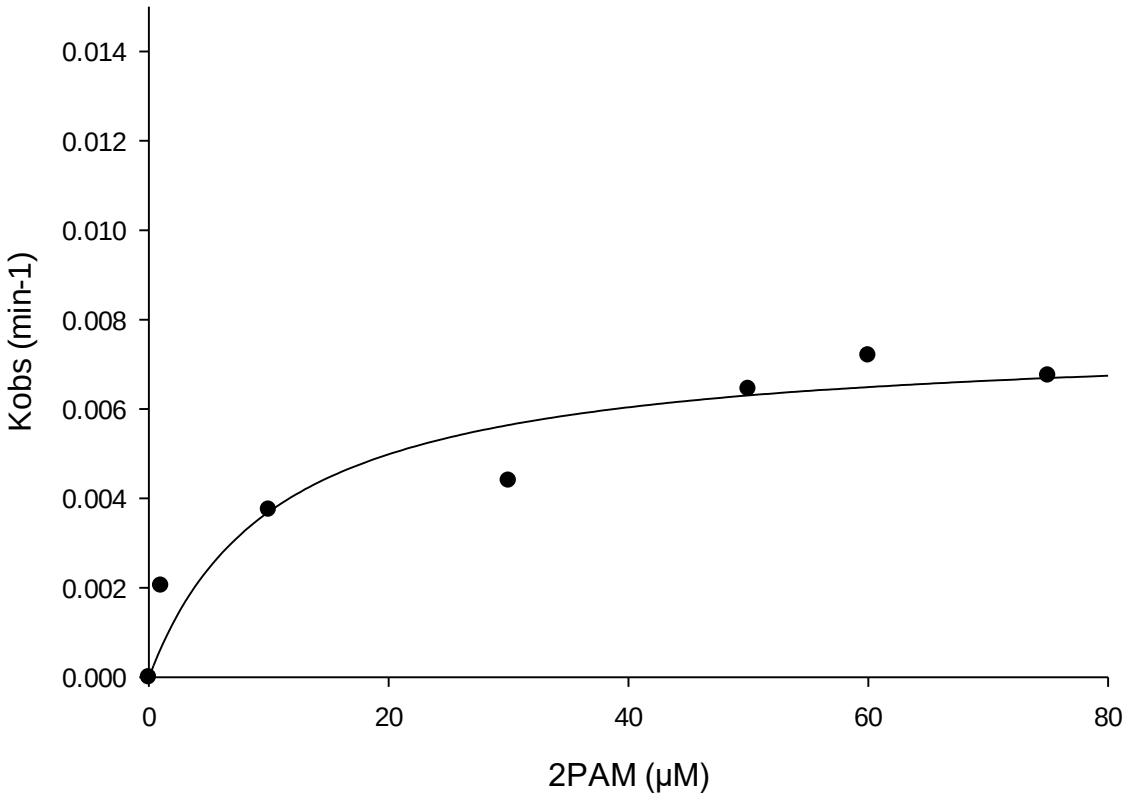



Figure 3.16 Plot of $k_{obs}$ for NIMP inhibited human serum BChE reactivated by various concentrations of 2PAM. $k_{obs}$ vs [2PAM] allowed the determination of the rate constant $k_r$ and affinity constant $K_D$. $R^2$ of 0.90

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CHAPTER IV

# INTRANASAL DELIVERY OF OXIMES FOLLOWING EXPOSURE TO A SARIN SURROGATE

## Introduction

Organophosphorus compounds (OPs) potently inhibit the serine hydrolase acetylcholinesterase (AChE). Its inhibition rapidly leads to an accumulation of the excitatory neurotransmitter acetylcholine (Ach) in the peripheral and central nervous systems. This increase in Ach leads to a persistent stimulation of nicotinic and muscarinic receptors which, as a consequence to high dosage exposures, can lead to respiratory distress, excitotoxic seizures, and death to the victim if treatment is not quickly administered. Treatment strategies consist of the antimuscarinic drug atropine sulfate, an oxime reactivator such as 2-PAM to remove the phosphoryl moiety from the inhibited enzyme, and an anticonvulsant such as diazepam to control seizures. Even if the victim survives, long term brain damage can still occur because of prolonged seizures if treatment is not quickly administered (Aroniadou-Anderjaska *et al.*, 2016). Research shows that several years after the nerve agent attack in the Tokyo subway system by the terrorist group Aum Shinrikyo (AUM), victims showed significant atrophy in multiple brain regions (Yamasue *et al.*, 2007; Yamasue *et al.*, 2003; Rogers *et al.*, 2009).

The proposed mechanism of seizure onset and maintenance in experimental animal models following organophosphate challenge has been suggested to consist of three distinct phases: the cholinergic phase (which lasts from the time of exposure to about 5 minutes after

seizure onset), the mixed cholinergic phase (which begins after 5 minutes and can last until 40 minutes), and the noncholinergic phase which begins around 40 minutes (McDonough and Shih, 1997). The initial cholinergic phase results from the OP induced inhibition of AChE and its subsequent decrease in its activity. This inhibition causes a persistent increase of Ach levels in the brain (Flynn *et al.,* 1986; Flynn *et al.,* 1987; Fosbraey *et al.,* 1990) which is an important initiator of seizure activity because the elevation of Ach levels causes an increase in excitatory activity of the brain that can spread and disrupt other neurotransmitter systems (McDonough and Shih, 1997). In fact, within the first few minutes of OP exposure and seizure onset, cholinergic activity is the only neurotransmitter system that is notably perturbed (McDonough and Shih, 1997). The mixed cholinergic and noncholinergic phases begin to occur 5-40 minutes after seizure onset and consist of other neurotransmitter systems and secondary messenger systems that are increased such as glutamate and calcium ($Ca^{++}$), respectively, which have the potential to be neurotoxic, and the decrease in activity of the inhibitory neurotransmitter GABA (McDonough and Shih, 1997).

In a high dose exposure of OPs, brain regions that display high AChE inhibition, such as the basolateral nucleus of the amygdala and the hippocampus, show significant neuropathology and brain damage from seizures induced by OPs (Aroniadou-Anderjaska *et al.,* 2016). These regions play important roles in memory, emotion, and cognitive function, and impairment of these areas leads to neurological and behavioral abnormalities. The current treatment strategy for attenuating seizures is administration of diazepam, a benzodiazepine. However, the use of diazepam has produced mixed results at attenuating neuropathology and brain damage even if seizures are stopped, and it poses health risks if repeated administrations are needed (Apland *et al.,* 2014; Goodkin *et al.,* 2008; Naylor, 2010).

Therefore, being able to rapidly attenuate AChE inhibition by oxime reactivator treatment is paramount to preventing the cascade of secondary events that can cause persistent seizures and brain damage. Unfortunately, the currently approved oxime reactivator therapeutics only act in the peripheral nervous system, so the central nervous system AChE remains inhibited (Gallagher *et al.*, 2016; Kalasz *et al.*, 2014; Worek and Thiermann, 2013; Lorke *et al.*, 2008; Sakurada *et al.*, 2003; Clement, 1979). A critical research goal for scientists has been to develop an oxime that has the ability to cross into the brain without losing reactivation capability. To bypass the blood brain barrier (BBB), chemists need to make oximes that are small and lipophilic, because of the BBBs ability to efflux large and small molecular weight compounds that are water soluble. It is estimated that the blood brain barrier prevents >98% of hydrophilic small and large molecules from crossing into the brain (Djupesland *et al.*, 2014; Pardridge, 2012). Therefore, to pass through the BBB, it is critical for a compound to be small and lipophilic.

To bypass the poor blood brain barrier penetration of oximes, delivery of the oxime intranasally, as opposed to intramuscularly, has been an emerging technique and achieved significant success at preventing CNS damage (Krishnan *et al.*, 2016).What makes intranasal delivery an effective method of administration is the rich vascular supply of the nasal cavity, thus providing a speedy delivery from nose to brain and therefore a potentially rapid onset of therapeutic effects. This vascular supply is primarily from the opthalamic artery, the sphenopalatine artery, and the facial artery (Lochhead and Thorpe, 2011). Transport into the brain from the nasal cavity bypasses the BBB, thus allowing molecules with various physicochemical characteristics entry into an otherwise restricted area. Absorption can occur via the olfactory or trigeminal pathway. The olfactory nerve has direct contact with various brain structures and the upper portions of the nasal cavity, providing a direct contact between the

environment and the brain (Djupesland *et al.*, 2014). The trigeminal nerve has innervations that arise from the pons and brainstem and project all the way to the nasal epithelium and olfactory bulb from its multiple branches (Lochhead and Thorpe, 2011). The processes of absorption into the brain can take place via different transport mechanisms, such as intracellular transport (i.e., being transported via anterograde transport on the axons of the olfactory and trigeminal nerve components via endocytosis) and extracellular transport (absorption from blood or lymph, or diffusion from perineural spaces into the brain) (Lochhead and Thorpe, 2011). Being able to administer an antidote that can bypass the BBB and provide a rapid therapeutic onset that can protect against CNS damage would be a huge upgrade to the current therapeutic regimen against OPs.

## Materials and Methods

### Chemicals

The organophosphate used for this *in vivo* study was the sarin surrogate nitrophenyl isopropyl methylphosphonate (NIMP). It was synthesized by the late Dr. Howard Chambers and originally described in Ohta *et al.* (2006) and characterized in our laboratories (Meek *et al.*, 2012). The novel oxime used in this study was oxime 20, also synthesized by the late Dr. Howard Chambers and originally described in Chambers *et al.* (2013). 2-pyridinium aldoxime (2-PAM), the currently FDA-approved oxime reactivator in the United States, was used as the comparison oxime and was purchased from Sigma Aldrich Chemical Company (St. Louis, MO).

### Animals

Adult male Sprague Dawley rats (Crl:CD(SD)BR) at postnatal day (PND) 70 were purchased from Envigo RMS, Inc. and housed in AAALAC accredited facilities at Mississippi State University. All animals were held in temperature-controlled environments with a 12-hour

light-dark cycle, used Envigo 70-90 Sani Chips, laboratory grade bedding and had free access to Envigo Rodent Diet (18% protein laboratory rat chow) and tap water. Procedures performed for this research received prior approval by the Mississippi State University Animal Care and Use Committee.

**Treatment Paradigm**

Four rats were used for this experimental paradigm. One rat received vehicle (Multisol, a biocompatible solvent consisting of 48.5% water, 40% propylene glycol, 10% ethanol, and 1.5% benzyl alcohol) to serve as vehicle control while three rats received a subcutaneous dose of NIMP. Each subcutaneous administration was delivered in the back of the neck as performed previously (Chambers *et al.*, 2016). One rat received NIMP alone while the other two rats were to receive intranasal oxime therapy. The dose of NIMP resulted in 70-75% brain AChE inhibition at 1hr following OP challenge (see Figure 4-1 for time course of NIMP brain AChE inhibition). This dose was determined to be 0.275mg/kg, which is a little lower than previous sub lethal studies performed in our laboratory of NIMP (0.3mg/kg) which resulted in 80-85% brain AChE inhibition at 1hr (Chambers *et al.*, 2013). Higher levels of inhibition would have produced severe cholinergic signs, making the intranasal procedure difficult to complete. This study wanted to determine proof of principle that intranasal delivery could consistently reduce brain AChE inhibition. The inhibition observed was high enough to observe a therapeutic benefit but low enough to allow for consistent and reproducible oxime intranasal delivery. Each challenge of NIMP was given 20 minutes apart. Five repetitions were completed for each treatment group.

**Intranasal Procedure**

At one hour post vehicle or NIMP administration, the animals were deeply anesthetized with 5% isoflurane and placed in the supine position. An intracatheter device (provided by Impel

NeuroPharma) was inserted into the nasal cavity where 10µl of either oxime (at a dose of 1.46µmoles/kg delivered in Multisol) or vehicle control (Multisol) was delivered bilaterally onto the olfactory epithelium in both nostrils.

**Tissue Collection and Preparation**

After the intranasal administration procedure was complete, animals were euthanized by decapitation at a nominal time zero (i.e., euthanized as quickly as possible), 5 minutes post oxime administration, or 10 minutes post oxime administration. The brains were quickly removed from the skull, washed with 0.9% saline and placed in a brain tissue dissection block. Brains were sliced coronally into three pieces: forebrain, midbrain, and hindbrain. These slices were snap frozen in liquid nitrogen and stored at -80°C until they were assayed. Brain slices were homogenized separately, and immediately prior to the assay, utilizing a motorized homogenizer in 50mM Tris HCl buffer (pH 7.4 25°C) at a stock solution of 40mg/ml wet weight equivalent and were maintained at 4°C.

**Measurement of Cholinesterase Activity**

AChE activity was measured in all brain slices by performing a continuous spectrophotometric essay. In a water bath (37°C), 100µl of brain homogenate was added to a test tube containing 1900µl of 50mM Tris HCl buffer (pH 7.4 25°C) for a final concentration of 1.8mg of homogenate/ml. Following this, 125µl of 5,5'-dithio bis(2-nitrobenzoic acid) (DTNB, FC 1.38mM) was delivered and the reaction was initiated by the addition of 50µl of acetylthiocholine (ATCh in EtOH, FC 2.3mM). The contents of the tube were vortexed thoroughly and immediately transferred to a cuvette and placed in a Thermo Scientific Biomate 3 spectrophotometer where absorbance values (A) were measured at 412nm and the slope was calculated from the absorbance measured over two minutes. The absorbance values are directly

proportional to the amount of substrate hydrolyzed by AChE. The percentage of AChE inhibition was calculated as described in chapter 2.

**Statistical Methods**

The effect of treatment, time, and brain section on absorbance was assessed using a generalized linear mixed model with PROC MIXED in SAS for Windows (SAS Institute, Inc., Cary, NC). Fixed effects included treatment, time, brain section, and all two-way and three-way interactions. Rat within replicate and replicate were included as random effects. In the case of a significant three-way interaction term, LSMESTIMATE statements were used to make pairwise comparisons between treatments at each combination of time and brain section, between time points for each combination of treatment and brain section, and between brain sections for each combination of time and treatment. The SIMULATE option was used to adjust p-values for multiple comparisons for each set of pairwise comparisons. The distribution of residuals was evaluated to assess if the assumptions of normality and homoscedasticity had been met. An alpha level of 0.05 was used to determine statistical significance.

**Results**

Results indicated that animals receiving novel oxime 20 showed a statistically significant difference in AChE absorbance in the forebrain and midbrain ($p<0.05$) when compared to NIMP alone treated animals and animals that received 2-PAM at the nominal time zero time point (Figure 4-2 and Figure 4-3). 2-PAM did not differ from NIMP alone treated animals at the nominal time zero time point for any of the brain regions, indicating limited entry into the brain. For the 5 minute time point, animals receiving either oxime 20 or 2-PAM showed a statistically significant difference ($p<0.05$) in AChE absorbance values when compared to NIMP alone treated animals in the forebrain (Figure 4-5) but no significant differences were detected between

the two oximes or in other brain regions (Figure 4-6 and Figure 4-7). No significant differences were detected in AChE absorbance values between the two oximes and NIMP alone treated animals during the ten minute time point in any brain region (Figures 4-8, 4-9, and 4-10).

## Discussion

To improve the amount of oxime that can enter into the brain and reactivate OP inhibited brain AChE, intranasal delivery of oximes was investigated as a potential alternate route of administration. Despite the limited number of animals used in this study, novel oxime 20 indicated rapid entry into the brain. Oxime 20 was statistically significantly different than NIMP alone treated animals and 2-PAM treated animals at the nominal time zero point, indicating more reactivation has occurred with oxime 20 and that less AChE inhibition is present. This could be due to the fact that oxime 20 is significantly more lipophilic than 2-PAM (Chambers *et al.*, 2013). This is further supported by the fact that by five minutes 2-PAM and novel oxime 20 absorbance values in the forebrain were not statistically different from each other but were statistically significantly different from NIMP alone treated animals. Interestingly, this was the only brain region that showed differences between NIMP alone treated animals in the 5 minute time point. Additionally, no significant differences were observed in any brain region when the animals were sacrificed at 10 minutes (Figures 4-8, 4-9, and 4-10) after oxime administration nor were significant differences observed in the hindbrain for any time point (Figures 4-4, 4-7, and 4-10). The lack of significant differences in the hindbrain could be explained by the fact that it is the most distal region from the site of administration (the olfactory bulb) and that most of the oxime has interacted with AChE in other brain regions or that the oxime was removed from the brain and entered into systemic circulation.

Nevertheless, we have demonstrated proof of principle that intranasal delivery can be a viable route of oxime administration to bypass the blood brain barrier. Both oximes entered into the brain within a couple of minutes while oxime 20 appeared to enter faster, indicating a wider window of effectiveness compared to 2-PAM. Future studies will need to investigate if multiple administrations of intranasal oxime delivery are well tolerated, thus potentially increasing the duration of oxime into the brain and if the more distal brain regions can be reached. In this study, a single oxime administration did not appear to be very effective as most of the data were not statistically significant. It is important to note that a limited number of animals were used and thus it is difficult to draw definitive conclusions. These initial, preliminary results are quite promising and warrant future investigations. Future experiments involving lethal doses of nerve agent, multiple oxime administrations, and neuropathology staining would be a better indicator of intranasal oxime effectiveness.

## Conclusion

Intranasal delivery of oximes would be of great value to medical and military personnel. The risk of long term brain damage after exposure to OPs is of significant concern. Being able to effectively attenuate the AChE inhibition in the brain, which is the first step to seizure onset, is paramount to preventing seizures and brain damage. The simplicity of use and ability to rapidly administer an oxime that can bypass the blood brain barrier and attenuate high AChE inhibition makes this treatment modality an attractive adjunct to the current therapeutic regimen. The use of intranasal oxime therapy in conjunction with an anticonvulsant could drastically improve CNS protection by providing multiple methods in treating OP induced neurotoxicity.

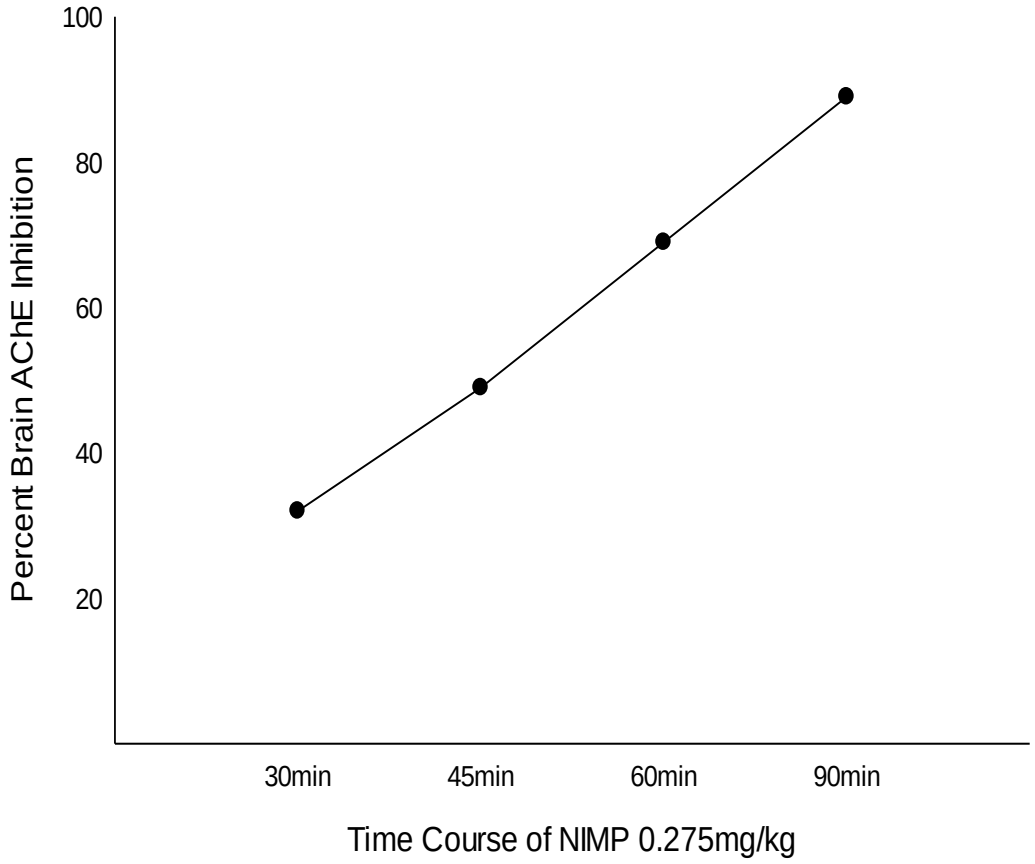


Figure 4.1 Brain acetylcholinesterase (AChE) inhibition time course of NIMP 0.275mg/kg delivered subcutaneously.

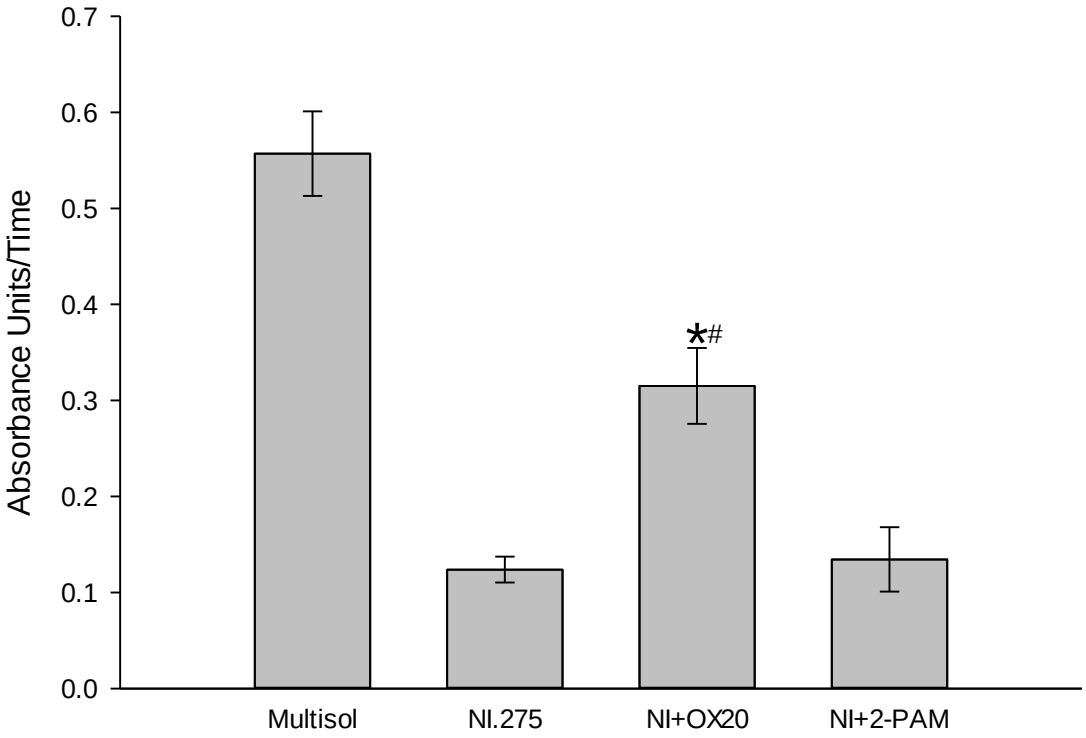


Figure 4.2 Acetylcholinesterase (AChE) absorbance of the forebrain following exposure to NIMP 0.275mg/kg (NI.275). Treatment groups are NIMP + oxime 20 (NI+OX20) and NIMP + 2-PAM (NI+2-PAM). Oximes were administered one hour post NIMP exposure and animals were sacrificed as quickly as possible to yield a nominal time zero. Data are expressed as mean ± SEM. N=5 animals for each treatment group. p*≤0.05 when compared to NIMP; p#≤0.05 when compared to NI+2-PAM.

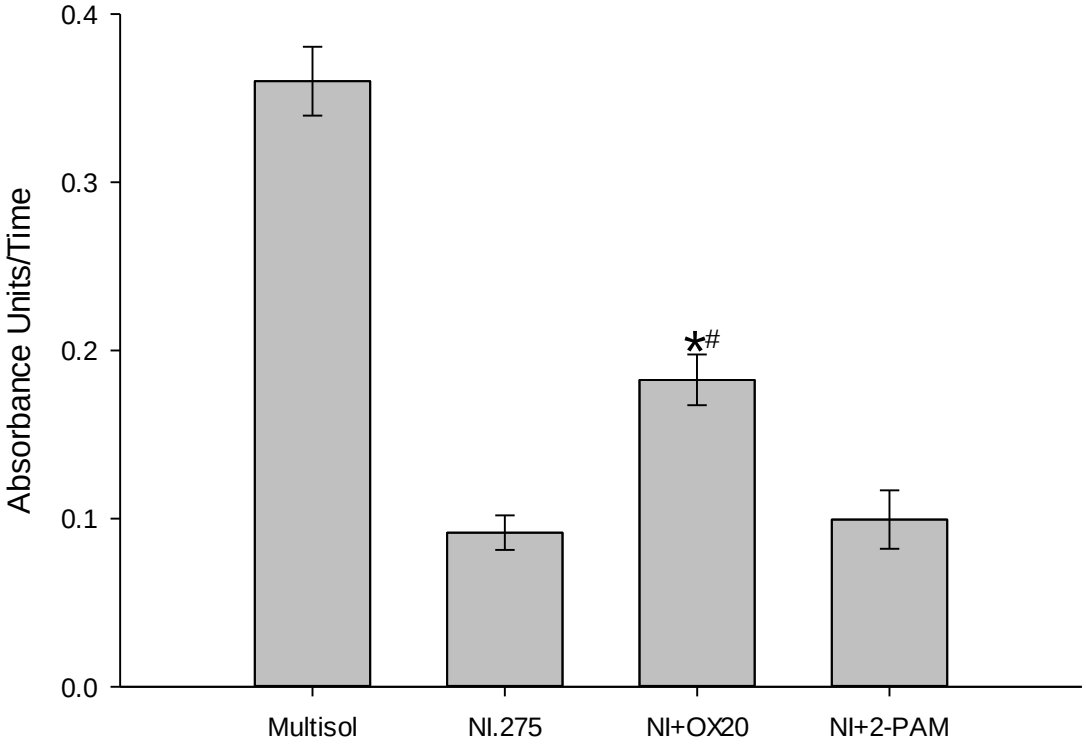


Figure 4.3 Acetylcholinesterase (AChE) absorbance of the midbrain following exposure to NIMP 0.275mg/kg (NI.275). Treatment groups are NIMP + oxime 20 (NI+OX20) and NIMP + 2-PAM (NI+2-PAM). Oximes were administered one hour post NIMP exposure and animals were sacrificed as quickly as possible to yield a nominal time zero. Data are expressed as mean ± SEM. N=5 animals for each treatment group. p*≤0.05 when compared to NIMP; p#≤0.05 when compared to NI+2-PAM.

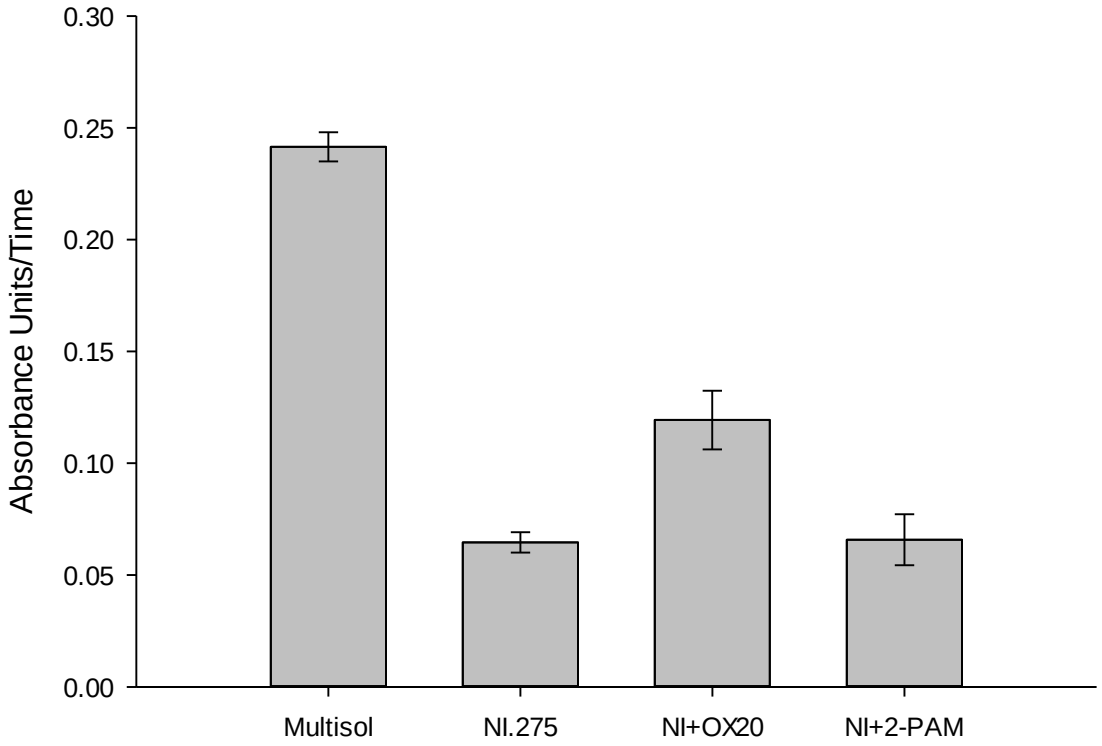


Figure 4.4 Acetylcholinesterase (AChE) absorbance of the hindbrain following exposure to NIMP 0.275mg/kg (NI.275). Treatment groups are NIMP + oxime 20 (NI+OX20) and NIMP + 2-PAM (NI+2-PAM). Oximes were administered one hour post NIMP exposure and animals were sacrificed as quickly as possible to yield a nominal time zero. Data are expressed as mean ± SEM. N=5 animals for each treatment group.

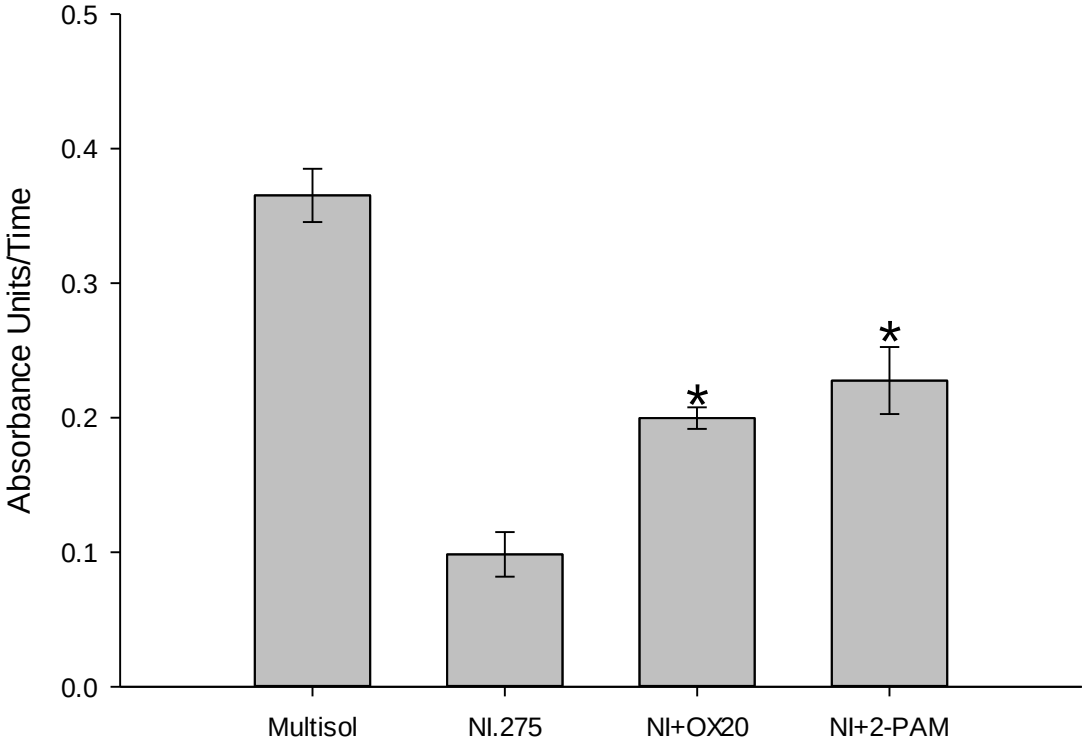


Figure 4.5 Acetylcholinesterase (AChE) absorbance of the forebrain following exposure to NIMP 0.275mg/kg (NI.275). Treatment groups are NIMP + oxime 20 (NI+OX20) and NIMP + 2-PAM (NI+2-PAM). Oximes were administered one hour post NIMP exposure and animals were sacrificed five minutes post oxime administration. Data are expressed as mean ± SEM. N=5 animals for each treatment group. $p^* \leq 0.05$ when compared to NIMP

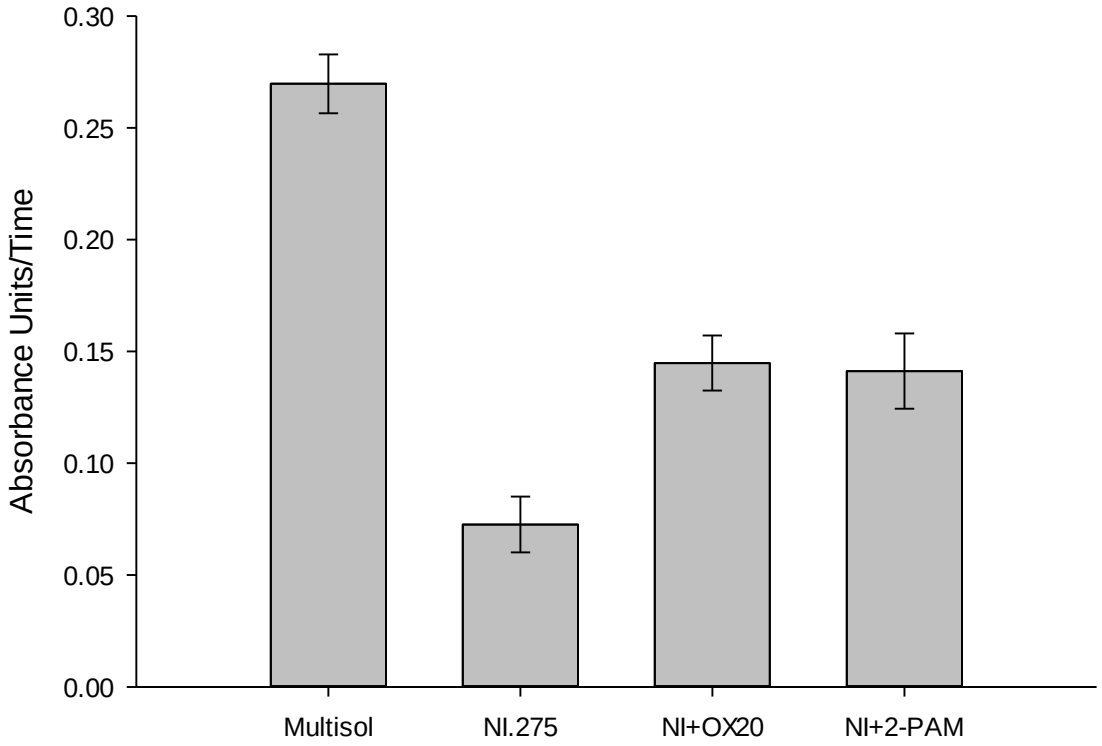


Figure 4.6 Acetylcholinesterase (AChE) absorbance of the midbrain following exposure to NIMP 0.275mg/kg (NI.275). Treatment groups are NIMP + oxime 20 (NI+OX20) and NIMP + 2-PAM (NI+2-PAM). Oximes were administered one hour post NIMP exposure and animals were sacrificed five minutes post oxime administration. Data are expressed as mean ± SEM. N=5 animals for each treatment group.

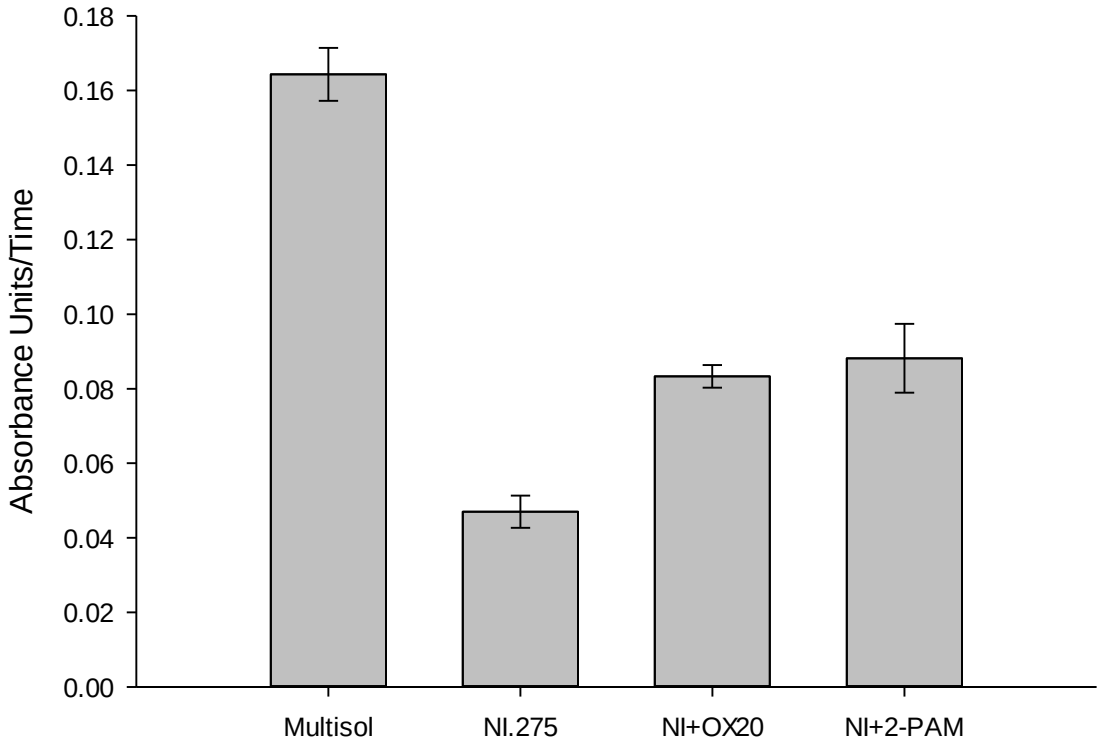


Figure 4.7 Acetylcholinesterase (AChE) absorbance of the hindbrain following exposure to NIMP 0.275mg/kg (NI.275). Treatment groups are NIMP + oxime 20 (NI+OX20) and NIMP + 2-PAM (NI+2-PAM). Oximes were administered one hour post NIMP exposure and animals were sacrificed five minutes post oxime administration. Data are expressed as mean ± SEM. N=5 animals for each treatment group.

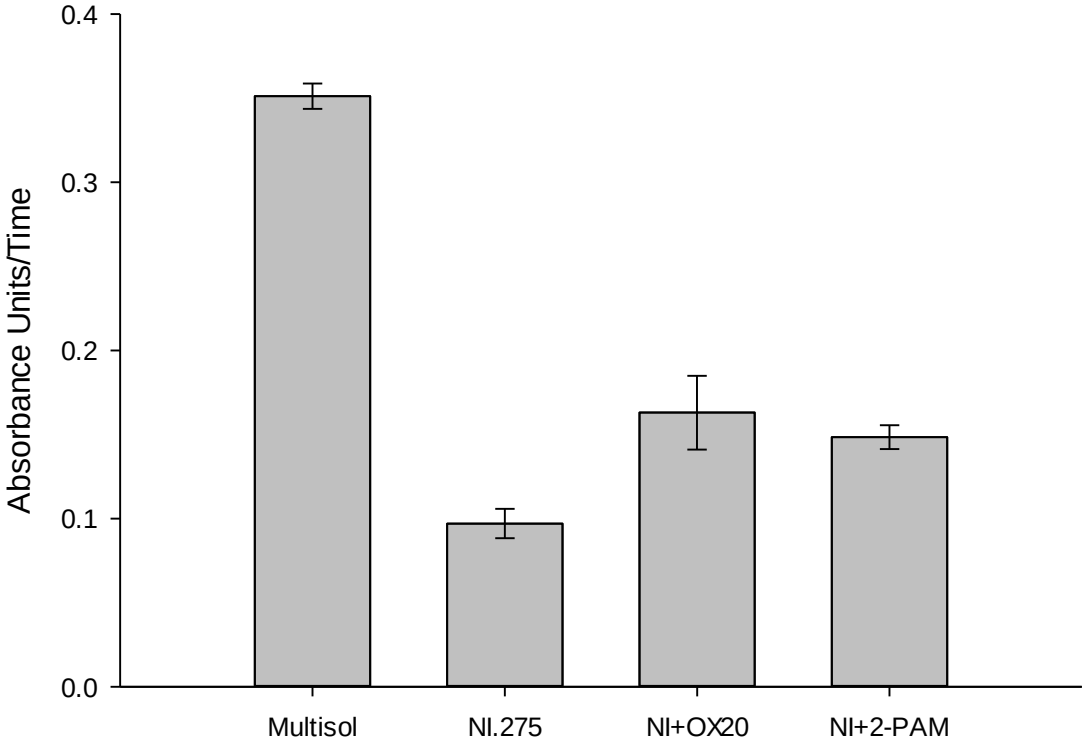


Figure 4.8 Acetylcholinesterase (AChE) absorbance of the forebrain following exposure to NIMP 0.275mg/kg (NI.275). Treatment groups are NIMP + oxime 20 (NI+OX20) and NIMP + 2-PAM (NI+2-PAM). Oximes were administered one hour post NIMP exposure and animals were sacrificed ten minutes post oxime administration. Data are expressed as mean ± SEM. N=5 animals for each treatment group.

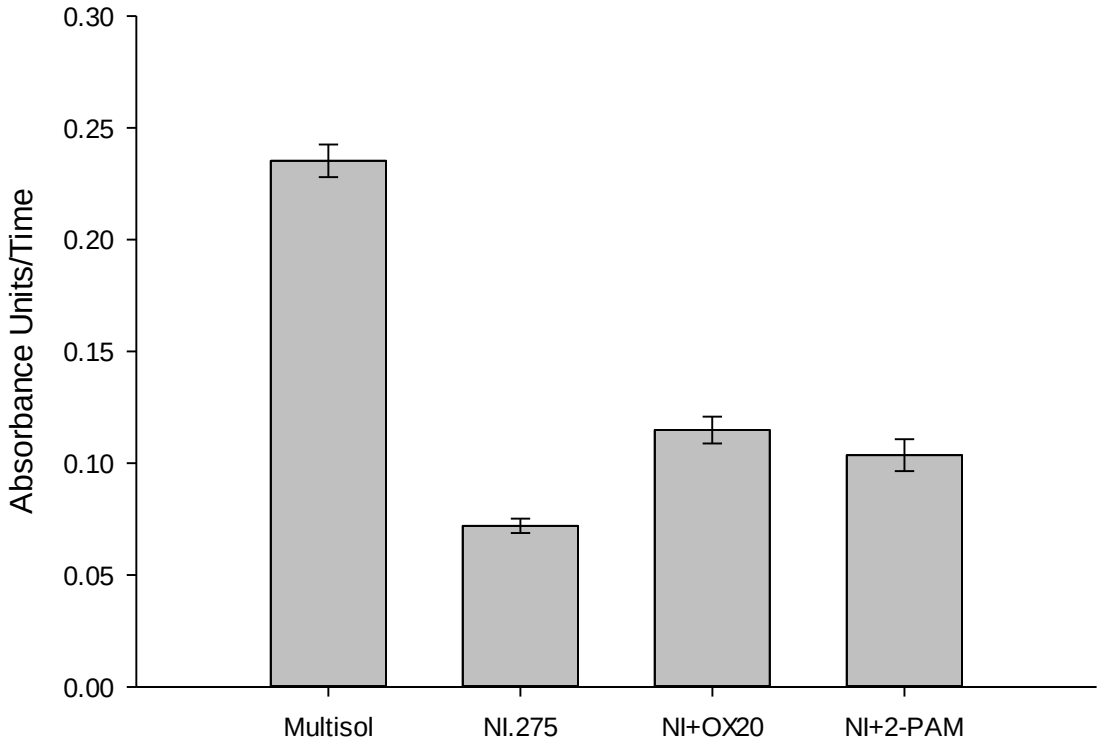


Figure 4.9 Acetylcholinesterase (AChE) absorbance of the midbrain following exposure to NIMP 0.275mg/kg (NI.275). Treatment groups are NIMP + oxime 20 (NI+OX20) and NIMP + 2-PAM (NI+2-PAM). Oximes were administered one hour post NIMP exposure and animals were sacrificed ten minutes post oxime administration. Data are expressed as mean ± SEM. N=5 animals for each treatment group.

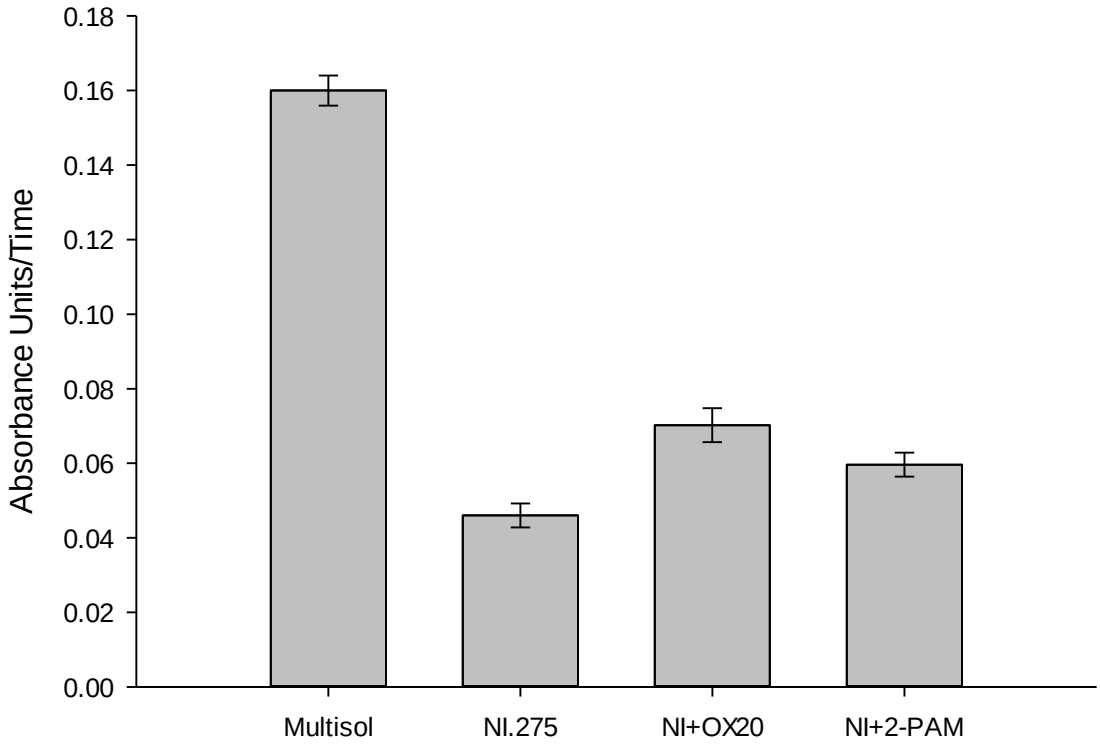


Figure 4.10 Acetylcholinesterase (AChE) absorbance of the hindbrain following exposure to NIMP 0.275mg/kg (NI.275). Treatment groups are NIMP + oxime 20 (NI+OX20) and NIMP + 2-PAM (NI+2-PAM). Oximes were administered one hour post NIMP exposure and animals were sacrificed ten minutes post oxime administration. Data are expressed as mean ± SEM. N=5 animals for each treatment group.

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# CHAPTER V

# CONCLUSION

Organophosphate pesticides represent a major public health problem and have been for several decades. Self ingestion of OP pesticides has represented a significant portion of suicide attempts in undeveloped countries for several decades. In India, from 2000-2002, OP pesticide poisoning accounted for 40.5% of committed suicides (Prasad *et al.*, 2006), while in Asia they represented two thirds of the self ingested pesticide cases (Eddleston, 2000). Unfortunately, this trend of poisoning continues to be a problem. Pesticide self ingestion, including self ingestion with OPs, continues to be the suspected cause of at least 200,000 deaths every year (Eddleston and Chowdhury, 2016). The diversity in OP pesticide structures, high body load of OP after self ingestion and ill equipped medical facilities in these areas present significant challenges to clinicians to effectively treat their patients.

Despite being banned for use in warfare, OP nerve agents are still used to carry out assassination attempts and to commit terrorist attacks. The use of nerve agents by the Syrian regime on the battlefield of Syria, the recent assassination of King Jong Nam by the North Korean government using the nerve agent VX, and the assassination attempt on a former Russian intelligence officer orchestrated by the Russian government using a new brand of nerve agent called Novichok indicate the willingness of nation states to use nerve agents to carry out their goals. The effectiveness of nerve agents and apparent lack of concern that governments have for international law can certainly bolster terrorist groups and other individuals to use such agents. Unfortunately, the widely available synthesis and development of some of the nerve agents in the

open literature (Holmstedt, 1951; Bryant *et al.,* 1960; Tammelin, 1957, Tammelin, 1957) poses significant security challenges for law enforcement and security personnel, making these compounds a likely threat for years to come.

Oxime reactivator treatment for OPs has remained largely unchanged. 2-PAM, HI-6, TMB-4, MMB-4, and obidoxime were synthesized in the 1950s and 1960s. 2-PAM, TMB-4 and obidoxime are the only oximes to have been used in a clinical setting despite thousands of oximes being synthesized since their inception. The poor blood brain barrier penetration and the lack of effectiveness against structurally different OPs among these current oxime platforms are well verified (Eyer and Worek, 2007; Musilek *et al.,* 2009; Worek *et al.,* 2010). Development of new oxime reactivators that are neuro-protective and broad spectrum are desperately needed to improve the antidotal treatment against OPs.

Our novel phenoxyalkyl pyridinium oximes have shown promise as brain penetrating reactivators of AChE to multiple OP chemistries, attenuating OP induced brain damage, seizure like activity, and increasing survival when compared to 2-PAM (Dail *et al.,* 2019; Pringle *et al.,* 2018; Chambers *et al.,* 2016, Chambers *et al.,* 2016, Chambers *et al.,* 2016, Chambers *et al.,* 2013). While the proposed mechanism of action of oximes is reactivation of AChE, research suggests that there are other non-direct mechanisms of oximes that can lead to a therapeutic benefit. Oxime interaction with cholinergic receptors is a verified phenomenon (Amitai *et al.,* 1980; Kuhnen-Clausen *et al.,* 1983; Kloog *et al.,* 1986) and has been suggested to play an important role in attenuating OP toxicity and lethality despite the evidence of low AChE reactivation (Melchers *et al.,* 1991; Van Helden *et al.,* 1991).

The primary goal of this research was to investigate an alternative oxime mechanism that may be contributing to the increased survival seen with our novel oximes. This research focused

on the reactivation of serum butyrylcholinesterase (BChE) as a potential novel therapeutic target. Reactivation of serum BChE could increase the detoxication of circulating OP molecules, thus less OP can reach critical target areas and cause toxicity. Additionally, this research investigated intranasal delivery of oximes as an alternative method of oxime administration. Traditionally, oximes are administered intramuscularly (IM). While IM administration of oximes is effective at reactivating peripheral AChE, very little oxime can enter into the brain and reactivate CNS inhibited AChE because of their inability to effectively cross the blood brain barrier. Delivery of the oximes intranasally can provide a novel way to allow more oxime into the brain and attenuate OP induced neurotoxicity.

In the first part of the study, a well verified reactivation assay was performed to assess oxime-mediated BChE reactivation *in vitro* in rat, human, and guinea pig serum. Several tested novel oximes were used and compared to the current oxime platforms 2-PAM, HI-6, obidoxime, TMB-4, and MMB-4 after inhibition by structurally different OPs. These OPs consisted of nerve agent surrogates for sarin, VX, and cyclosarin and of the insecticidal OP oxons paraoxon, phorate oxon, and phorate oxon sulfoxide. Data from these experiments revealed several effective BChE reactivators. Novel oxime 15 was determined to be the most effective for OP inhibited rat BChE. Demonstrating >75% reactivation after inhibition by the sarin surrogate PIMP, the VX surrogate NEMP, and paraoxon. Novel oxime 20 was determined to be the most effective oxime for OP inhibited human BChE. Oxime 20 demonstrated impressive broad spectrum efficacy towards 6 of the 8 OP compounds tested, reaching greater than 70% reactivation for sarin and VX surrogates, and the phorate metabolites (phorate oxon and phorate oxon sulfoxide). 2-PAM, TMB-4, MMB-4, obidoxime and HI-6 were poor BChE reactivators. All oximes were poor reactivators of OP inhibited guinea pig BChE.

The second part of this study investigated oxime reactivation kinetics for novel oxime 15, novel oxime 20, and 2-PAM for electric eel AChE and rat and human serum BChE after inhibition by the sarin surrogate NIMP and the insecticidal metabolite paraoxon. Results indicated that both novel oxime 15 and oxime 20 were more effective reactivators of NIMP and paraoxon inhibited electric eel AChE when compared to 2-PAM, indicated by the $k_{r2}$. Additionally, novel oxime 20 was a more effective reactivator of NIMP inhibited human BChE than both novel oxime 15 and 2-PAM. While the intrinsic reactivity of our oximes was lower for rat inhibited BChE compared to the other species, the oximes displayed high affinity for the paraoxon inhibited enzyme ($K_D$ less than 1μM) and thus proved to be efficient reactivators. Oxime 20 also demonstrated efficacy towards NIMP inhibited rat BChE while oxime 15 and 2-PAM did not. Oxime 20 demonstrated an ability to reactivate both NIMP and paraoxon inhibited AChE and BChE.

The third part of this study investigated intranasal delivery of oximes as an alternative route of administration to treat OP inhibited brain AChE. This study demonstrated that intranasal delivery of either novel oxime 20 or 2-PAM were able to reduce NIMP inhibited brain AChE in select brain regions within a few minutes. Oxime 20 reached the brain faster than 2-PAM, indicating a wider range of effectiveness. However, neither oxime was able to effectively attenuate brain AChE inhibition in more distal brain regions such as the hindbrain. Nevertheless, the preliminary results of this study showed proof of principle that intranasally delivered oximes could be an effective route of administration to attenuate brain AChE inhibition.

The data presented in this study demonstrated that our novel oximes were more effective BChE reactivators than the current oxime therapeutics. These data could explain the increased survivability and cessation of seizure like behavior that was seen with novel oxime 20 *in vivo*

when compared to 2-PAM after exposure to lethal levels of sarin and VX surrogates and paraoxon in the rat model (Chambers *et al.*, 2016). Novel oxime 20 was a much more effective reactivator of sarin surrogate-, VX surrogate-, and paraoxon-inhibited rat BChE than 2-PAM (see Chapter 2). This increase in BChE reactivation should theoretically detoxify additional OP molecules in the circulation, thus a smaller amount of OP can enter into the brain and cause seizures and inhibit important respiratory control centers. It has also been demonstrated that oxime 20 can reactivate AChE (see Chapters 3 and 4). It may be the case that this dual capability of reactivating both AChE and BChE may be an important mechanism contributing to the therapeutic benefit of oxime 20. While more studies need to be completed to more accurately investigate the efficiency of AChE and BChE reactivation by oxime 20 in other animal models and human enzyme, as well as the efficacy of intranasally delivered oximes, both of these alternative mechanisms show promise as potential alternatives to increase the neuroprotection and broad spectrum capabilities of oxime reactivators that are desperately needed for treating OP nerve agents and insecticides.

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