1-1-2009

Assay of the Efficacy of Novel Pyridinium Oximes for Potential Activity in the Central Nervous System for Reactivating Phosphorylated Acetylcholinesterase

Ashley Renee Harmon

ASSAY OF THE EFFICACY OF NOVEL PYRIDINIUM OXIMES FOR POTENTIAL ACTIVITY IN THE CENTRAL NERVOUS SYSTEM FOR REACTIVATING PHOSPHORYLATED ACETYLCHOLINESTERASE

By

Ashley Renee Harmon

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Sciences
in the Department of Basic Sciences

Mississippi State, Mississippi

August 2009

ASSAY OF THE EFFICACY OF NOVEL PYRIDINIUM OXIMES FOR POTENTIAL ACTIVITY IN THE CENTRAL NERVOUS SYSTEM FOR REACTIVATING PHOSPHORYLATED ACETYLCHOLINESTERASE

By

Ashley Renee Harmon

College of Veterinary Medicine

Mississippi State University

Approved:

Janice E. Chambers William L. Giles Distinguished Professor Department of Basic Sciences Director of Thesis	Russell L. Carr Associate Professor Department of Basic Sciences Committee Member
Howard Chambers Professor Department of Entomology and Plant Pathology Committee Member	Larry Hanson Professor Director of Graduate Studies in the Department of Basic Sciences
Kent H. Hoblet Dean of the College of Veterinary Medicine	

Name: Ashley Renee Harmon

Date of Degree: August 2009

Institution: Mississippi State University

Major Field: Veterinary Medical Science (Toxicology)

Major Professor: Dr. Janice Chambers

Title of Study: ASSAY OF THE EFFICACY OF NOVEL PYRIDINIUM OXIMES FOR POTENTIAL ACTIVITY IN THE CENTRAL NERVOUS SYSTEM FOR REACTIVATING PHOSPHORYLATED ACETYLCHOLINESTERASE

Pages in Study: 40

Candidate for Degree of Master of Science

The aim of this research was to determine whether novel pyridinium oximes synthesized to increase lipophilicity and the likelihood of crossing the blood-brain barrier could effectively reactivate phosphorylated AChE *in vitro*. A synthesized sarin analog, phthalimidyl isopropyl methylphosphonate (PIMP), was used to test the reactivation potential of the oximes. The reactivation activities of the oximes on PIMP exposed AChE and structure activity relationships were examined. Differences in reactivation potential in comparison to the widely used 2-PAM were also examined. All novel oximes tested demonstrated some ability to reactivate inhibited AChE. Reactivations varied among the oximes (24%-78%), and were not effective as 2-PAM or TMB-4, 91-97%, respectively. The lipophilicity for all oximes was greater than 2-PAM or TMB-4 by 3 to 374 fold. A few of the novel oximes showed combined higher lipophilicity and reactivation potential approaching that of 2-PAM, and therefore suggest some potential efficacy as brain-penetrating oxime reactivators.

DEDICATION

I wish to dedicate this thesis to my parents who always believed in me and Dr. Janice Chambers for the faith she put in me.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God for giving me the strength and wisdom for believing in myself to complete this project. It sometimes got hard, but he always showed me the light at the other end of the tunnel.

I would like to give a big thanks to Dr. Jan Chambers for all of her guidance, support, encouragement, sacrifices, and editing which was immeasurably helpful. This has been an opportunity of a lifetime and I am more than grateful for it. I would like also to thank her for supporting and funding my graduate education, the discussions on my research, and lastly always being there when I needed her. She has truly made me a better person and prepared me for the next level in life.

I would like also to give a special thanks to Dr. Howard Chambers for his guidance and direction throughout my Master's program and thesis process. Thanks for all the help and answering my crazy questions. This project wouldn't be possible if it wasn't for his great mind.

I would like to thank Dr. Russell Carr for always putting a smile on my face with his humor. I would also like to thank him for serving on my committee and sharing his knowledge with me on various topics of my research.

A very special thanks goes to Edward Meek, MaryBeth Dail, Carole Nail and the basic science department. I wouldn't have made it without you guys. You all are my personal geniuses.

Lastly, I would like to thank my father for his patience, understanding, and being my support system. Love you dad. Mom thanks for being a good friend and a shoulder to lean on. Your prayers were answered. Thank You All and God Bless!!!

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Organophosphates (OPs) were first synthesized in the early 1800's when Lassaigne reacted ethanol with phosphoric acid to obtain triethyl phosphate. A hundred years later in 1936, Gerhard Schrader of Germany discovered nerve agents when trying to develop new types of insecticides. Schrader discovered the nerve agent tabun and subsequently discovered sarin in 1937. These powerful agents would later be classified as weapons of mass destruction by the United Nations Resolution 687 in 1991 (Resolution 687). Despite intensive endeavors by people around the world, some countries continue to keep nerve agents stockpiled which pose a potential threat to military forces and civilian populations. This research is aimed at taking considerable efforts to develop effective medical countermeasures for those potential threats.

Nerve agents and organophosphate insecticides are very similar and general treatment strategies are alike. The toxic actions of nerve agents are similar to one another, although there are differences between OPs with respect to their peripheral and central effects (Ligtenstein, 1984; Misulis *et al*., 1987). Nerve agents are a group of lipophilic and volatile OPs that have high acute mammalian toxicity, and are called nerve agents because of their effect on the CNS. The general formulae of nerve agents are very much alike those of OP pesticides, Figure 1. Nerve agents are the most toxic and rapidly acting of the known chemical warfare agents. The most important chemical reaction that occurs

with nerve agents takes place on the phosphorus atom, where the P-X bond can be easily broken by nucleophilic reagents, such as water or hydroxyl ions. In aqueous solutions at neutral pH, nerve agents slowly decompose. Alkali can accelerate this reaction (Weapons, 2005). Nerve agents may take the form of gas, aerosol, or liquids. Evidence of exposure may not be perceived by an individual until signs or symptoms occur, because nerve agents in their purest form are colorless, odorless, and tasteless. They can be inhaled or absorbed through the conjunctiva of the eye or through the skin. Nerve agents in liquid form can be absorbed through the gastrointestinal tract (Grob, 1953).

The main target of nerve agents is the enzyme acetylcholinesterase, acetylcholine acetyl hydrolase, EC 3.1.1.7, (AChE), a key component in the functioning of cholinergic brain synapses and neuromuscular junctions (Ecobichon, 1996). Nerve agents inhibit AChE by phosphorylating the active site of the enzyme. Phosphorylation of the active site inhibits the ability of AChE to hydrolyze the neurotransmitter acetylcholine (ACh). The accumulation of ACh causes over-stimulation of cholinergic synapses via muscarinic and nicotinic acetylcholine receptors (Abdel-Rahmanl *et al*., 2002). The accumulation causes hyperactivity in both the parasympathetic and sympathetic divisions of the autonomic nervous system. This excessive stimulation leads to autonomic nervous system effects, tremors, ataxia, convulsions, coma, and even death (Ecobichon, 2001). Nerve agent-exposed victims usually die from respiratory failure because their respiratory muscles become paralyzed by the action of nerve agents.

AChE is important because it is responsible for the hydrolytic degradation of the neurotransmitter ACh, which is involved in the transmission of nerve impulses to effector cells at neuromuscular, and cholinergic synaptic junctions. AChE consists of six active

sites, where each site has a central esteratic site and a peripheral anionic site (Nair and Hunter, 2004). The esteratic site of AChE is composed of a catalytic triad of serine, histidine and glutamic acid residues. These three acid residues perform the catalytic functions of the enzyme. OPs phosphorylate the serine hydroxyl group in the esteratic site of the enzyme (Marrs, 2007). The active site's esteratic and anionic sites are involved in the hydrolysis of ACh to acetic acid and choline. The anionic site of AChE is predicted to be composed of multiple negative charges and attracts the positive charge present on the nitrogen atom of ACh (Nolte *et al*., 1980). The nitrogen of ACh is oriented in a way that the carbonyl group is presented to the esteratic site and an enzyme-substrate complex is formed .The esteratic site recognizes and binds ACh. Electron shifts occur at the esteratic site with the result in acetylation of the serine at the active site of the enzyme and choline is released (Marrs, 2007). The acetylated enzyme complex is then hydrolyzed, freeing the enzyme and releasing acetic acid and returning the enzyme to its original state, Figure 2 (Soreq *et al*., 1993).

As stated earlier, differences between OPs may exist with respect to their relative central and peripheral effects (Ligtenstein, 1984; Misulis *et al*., 1987), i.e., there are differences in the sensitivity of AChE within the nervous system in the case of soman (Sellström *et al*., 1985). AChE sensitivity can be determined by an IC50, i.e., the concentration of inhibitor that inhibits 50% of AChE activity *in vitro.* AChE IC50 values reflect the intrinsic sensitivity of the AChE molecule to the inhibitor, and can be used to predict acute toxicity levels of OPs (Mortensen et al., 1998). Many researchers have conflicting evidence that acute toxicity as measured by LD_{50} correlates well with the target enzyme sensitivity measured by IC50 (Chambers *et al*., 1990; Johnson and

Wallace, 1987; Benke and Murphy, 1975), while other researchers are concerned about the interpretation of the IC50 value and suggested that the bimolecular rate constant (k_i) is a better tool for predicting OP toxicity. When one compares IC50 values it is important to keep in mind that experimental conditions must be identical, or the results are meaningless (e.g., tissue concentration, pH, temperature, assay, and incubation time). When interpreting IC50 values it should also be kept in mind that *in vitro* IC50 values are more indicative of the tissue's buffering capacity rather than representing the sensitivity of the AChE to inhibition by OPs (Mortensen et al., 1998). Furthermore, IC50 values do not give a true representation of the total kinetic reaction that occurs between OPs and AChE, although they reflect the potency of OPs (Richardson, 1999).

A phosphorylated enzyme may undergo either spontaneous reactivation or aging. Aging occurs from the result of a non-enzymatic loss of an alkyl group from the phosphorous atom, Figure 3. The rate of aging depends on the structure of the inhibited enzyme produced with each OP, e.g., soman phosphorylates the active site serine of AChE with a pinacolyl methylphosphonyl structure making the inhibition irreversible because of the loss of the pinacolyl group, Figure 4. Aging prevents both spontaneous and chemical induced reactivation of phosphorylated AChE stabilizing the phosphate-enzyme complex (Worek *et al*., 2007). The time course of aging is compound dependent, with a compound such as soman displaying very rapid aging. When comparing OPs such as sarin and soman, rat brain AChE aging half-life differs. Harris *et al*. (1966) report soman aging half-life as 0.037hr and Fleisher and Harris (1965) report sarin aging half-life as 5.8hr showing that aging is compound dependent and the rate of aging differs among OPs. When phosphorylated AChE has aged, it can be considered irreversible, but

the phosphorylated enzyme can be replaced through synthesis of a new enzyme, but this may take days. Phosphorylated AChE cannot be replaced in red blood cells and other formed elements of the blood that lack capacity for protein synthesis (Ecobichon, 2001; Wilson *et al*., 1992). When it can occur, spontaneous reactivation is an extremely slow process (10^7 to 10^{12} fold slower than deacetylation) that involves the dephosphorylation of the enzyme-inhibitor complex and the breaking of a covalent bond with the addition of water (Hobbiger, 1951; Johnson *et al.,* 2000). However, the amount of this dissociation complex present *in vitro* differs among OP compounds (Burgen and Hobbiger, 1951).

The oximes that are being used in this research are newly designed pyridinium oximes. Pyridinium oximes are formulated as different salts, i.e., iodide, chloride, lactate, bromide; they mostly differ in their stability and solubility (Antonijevic and Stojiljkovic 2006). Pyridinium oximes are compounds containing the group RCH=NOH attached to a pyridinium ring with a quaternary nitrogen that is capable of reactivating OP- inhibited AChE by dephosphorylating the active site of AChE (Lorke *et al*., 2008). Pyridinium oximes are oriented proximally to exert a nucleophilic attack on the enzyme inhibitor complex. The intermediate in the reactivation is a complex between the phosphorylated enzyme and the reactivator, in which the enzyme inhibitor-oxime complex splits off leaving a regenerated enzyme (Antonijevic *et al*., 2006). The potency of oximes such as 2-PAM is attributed to the quaternary nitrogen that binds to the anionic site of AChE and the positioning of the nucleophilic oxime moiety that supports the move of the phosphate from the active site of AChE to the oxime (Hackley *et al*., 1959).

Organophosphate poisoning resulting in the inhibition of AChE traditionally has been treated with atropine sulfate and an oxime, such as 2-PAM. Atropine antagonizes

the overstimulation of cholinergic receptors from accumulated ACh. Atropine is highly effective in antagonizing ACh receptors at muscarinic receptors, but is not as effective at nicotinic receptors, Figure 5. When used at the recommended dose levels atropine will act as antagonist at muscarinic receptors in the CNS, preventing convulsions and inhibition of the respiratory center (Johnson *et al.,* 2000). The role of an oxime is to free the inhibited enzyme from the OP and restore the enzyme function, Figure 3. The ability of pyridinium oximes to reactivate inhibited AChE can be influenced by a number of things: the number of quaternary pyridinium rings on the oxime (Hammond *et al.*, 2003; Kuca and Kassa, 2003); the shape and length of the chain that connects two quaternary nitrogens (Kuca *et al*., 2003); and the position and number of oxime groups at the pyridinium ring (Lamb *et al.*, 1966; Kuca and Kassa, 2003). Oximes display different characteristics such as oxime affinity and oxime reactivity which in turn can be used to design better oximes. All of the above can determine the efficacy of an oxime, but the therapeutic efficacy of oximes depends on many factors. It is difficult to treat OP poisoning, and factors such as timing and the administration route for both the nerve agent and antidote play a role.

Anticonvulsant drugs such as diazepam are also used in organophosphate poisoning to reduce seizures and convulsions. The immediate treatment with atropine sulfate and oxime does not always prevent nerve agent induced seizures. Prolonged seizures as a result of organophosphate poisoning can produce irreversible brain damage (McDonough *et al*., 1995; Shih *et al*., 2003). This can result in long term deficits in cognitive function and behavior (Shih *et al*., 2007). In most cases of OP poisoning the

central effects are mainly the results of activation of muscarinic receptors, and are antagonized by atropine (Rang *et al.,* 1999).

When determining reactivation constants of inhibited AChE with various nerve agents, there are cross-species differences, e.g., human, guinea pig, rat, and rabbit that show differences in the potency of oximes between human and animal enzymes. This may be due to enzyme-kinetic properties of AChE, toxicokinetics of inhibitors (Benschop and de Jong, 2001), or pharmacokinetics and dosing of antidotes (Baggot, 1994; Clement *et al*., 1995; Dawson, 1994) that interfere with the extrapolation of animal data to humans. It is known that all oximes can be toxic at a certain dose and should be given in recommended doses when administered.

It should not be assumed that nerve agents cannot cause much damage at doses lower than the lethal dose. People such as farm workers and factory workers who are exposed to OPs can suffer from long term low dose exposure. These workers who are exposed to excessive exposure to OPs can observe effects such as anxiety, agitation, headaches, impaired memory, difficulty in concentration, and psychiatric effects (Marrs, 2007). Military personnel that might be exposed to higher doses of OPs during battle might exhibit effects such as edema, necrosis in all cell-types, and respiratory failure that can eventually lead to death. A variety of effects in humans and animals have been attributed to OP exposure but it is unclear whether or not low doses can bring about long-term change in CNS function (Marrs, 2007).

It is more urgent now than it has been in the past to develop and improve the efficacy of oxime therapeutics. Currently available oximes are not ideal reactivators mainly due to their inability to penetrate the blood-brain barrier (BBB) and reactivate

AChE in the central nervous system because they are very hydrophilic. In addition it is known that currently available oximes are not equally effective in reactivating AChE that is inhibited by different OPs. Existing oximes can allow OP poisoning victims to survive, by reactivating the phosphorylated AChE in the peripheral nervous system, while the brain AChE remains considerably inhibited for an extended period of time. The design of oximes to penetrate the BBB involves increasing the lipophilicity of the oxime in relation to the cell membrane lipid bi-layer, i.e., endothelial cells (Tsuji, 1998). The blood-brain barrier is formed by endothelial cells of blood capillaries. The endothelial cells form tight junctions with adjacent cells, which subsequently form tight seals between the cells. Tight junctions are a manifestation of the blood-brain barrier, but the features of the brain microvascular endothelium also contribute to the limited and selective transport of toxicants between the blood and the brain (Ge *et al*., 2005). The tight junction prevents the diffusion of polar compounds through paracellular pathways, but there are other features of the brain microvascular endothelium that contribute to the limited and selective transport of substances between the blood and the brain. These transporters constitute a biochemical gatekeeper that ensures active regulation and nutrient delivery of the brain extracellular fluid, while preventing the entry of xenobiotics (Shujun *et al*., 2005). The transporters belong to a group of ATP- binding cassette transporters, and P-glycoprotein is the best characterized member of this protein family (Lorke *et al*., 2008). P-glycoprotein is known to be a gatekeeper in the blood-brain barrier by limiting the entry of toxic compounds. P-glycoprotein is located directly in the membrane of endothelial cells that forms the lipophilic barrier, making it capable of actively pumping compounds back into the blood (Schinkel, 1999).

It is believed that due to the virtual absence of transcytosis, lack of fenestrations and unique properties of tight junctions in brain endothelial cells, that the BBB only allows free diffusion of small lipophilic molecules (Lorke *et al*., 2008). The BBB is equipped with a variety of translocation and uptake systems for hydrophilic compounds such as amino acids. It is suggested that oximes that have either neutral or basic affinities for amino acids can penetrate the BBB by one of two carrier sites in which amino acids are transported, which are highly expressed in the BBB (Richter and Wainer,1971; Schinkel, 1999). Much is still unknown about the BBB, but it would be important to understand the mechanism of the BBB transport system in order to improve drugs that act in the CNS.

The aim of this research was to determine whether a series of novel pyridinium oximes, designed to be lipophilic enough to potentially cross the blood-brain barrier, were effective reactivators of phosphorylated AChE *in vitro*. The oximes along with two well known oximes, 2-PAM and TMB-4, were evaluated *in vitro* to determine the ability of the oximes to reactivate AChE in rat brain, Figure 6. 2-PAM is a standard therapeutic in the United States and the most well known oxime. 2-PAM reactivates phosphorylated AChE by nucleophilic attack on the serine residue removing the phosphate moiety. In previous studies 2-PAM has been reported to penetrate the blood-brain barrier somewhat, although there were numerous cases of civilians dying by sarin intoxication after 2-PAM was administered in the 1995 sarin attack in the Tokyo subway (Ohta *et al*., 2006). Nagao *et al*. (1997) found that when using the cortex and whole blood of two victims of the Tokyo attack as brain and blood samples, the victims' plasma AChE activities, using the Ellman method, were recovered by 2-PAM administration, but brain AChE activities

were not recovered in the victims (K. Sakurada *et al*., 2003). Trimedoxime (TMB-4) is a cholinesterase reactivator that reactivates AChE better than 2-PAM, but is more toxic, Figure 7.

Two organophosphate compounds were studied in this research to test the efficacy of the newly developed pyridinium oximes, paraoxon and a sarin analogue that is reflective of the nerve agent sarin. Paraoxon was used to screen the novel oximes to select the oximes to test with the sarin analogue, Figure 8. Paraoxon is an organophosphate, and the metabolite of the insecticide parathion. Paraoxon is used in many *in vitro* studies as an AChE inhibitor because of its high inhibitory potency. The experimental OP, phthalimidyl isopropyl methylphosphonate (PIMP), phosphorylates AChE with the same moiety as the nerve agent sarin, but degrades quickly in aqueous solution to prevent re-inhibition of reactivated AChE, Figure 9. PIMP will degrade in 15 minutes, thereby allowing the inhibition phase to cease. PIMP is non-volatile compared to sarin the most volatile of the nerve agents. Sarin changes from liquid to vapor very rapidly posing a short-lived threat. PIMP is a stable and nonvolatile analogue of sarin.

The reactivation activities of the pyridinium oximes on PIMP-exposed AChE and structure activity relationships were examined. The reactivation potential of the oximes was measured spectrophotometrically using a modification (Chambers *et al*., 1988) of the most widely used Ellman method (Ellman *et al*., 1961). Three replications of each oxime with each OP were performed. Whole rat brain was used as the source of AChE. The lipophilicities of the oximes were also determined by octanol-water partition coefficients, which is the simplest predictor of potential ability to cross the blood-brain barrier.

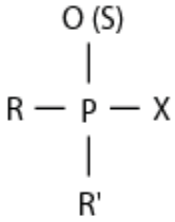


Figure 1 The general formulae for nerve agents and organophosphates.

R= alkyl, alkoxy, or aryl group, X= leaving group
Nerve agents are never P=S

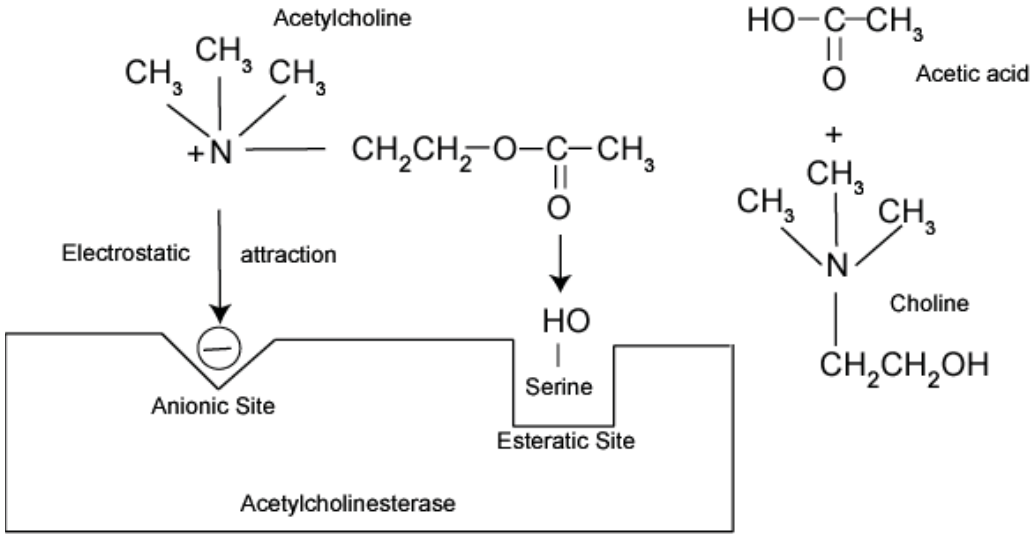


Figure 2 The normal function of acetylcholinesterase showing anionic and esteratic sites.

The positively charged nitrogen on the acetylcholine molecule is attracted to the anionic site on acetylcholinesterase, and hydrolysis is catalyzed at the esteratic site to form choline and acetic acid

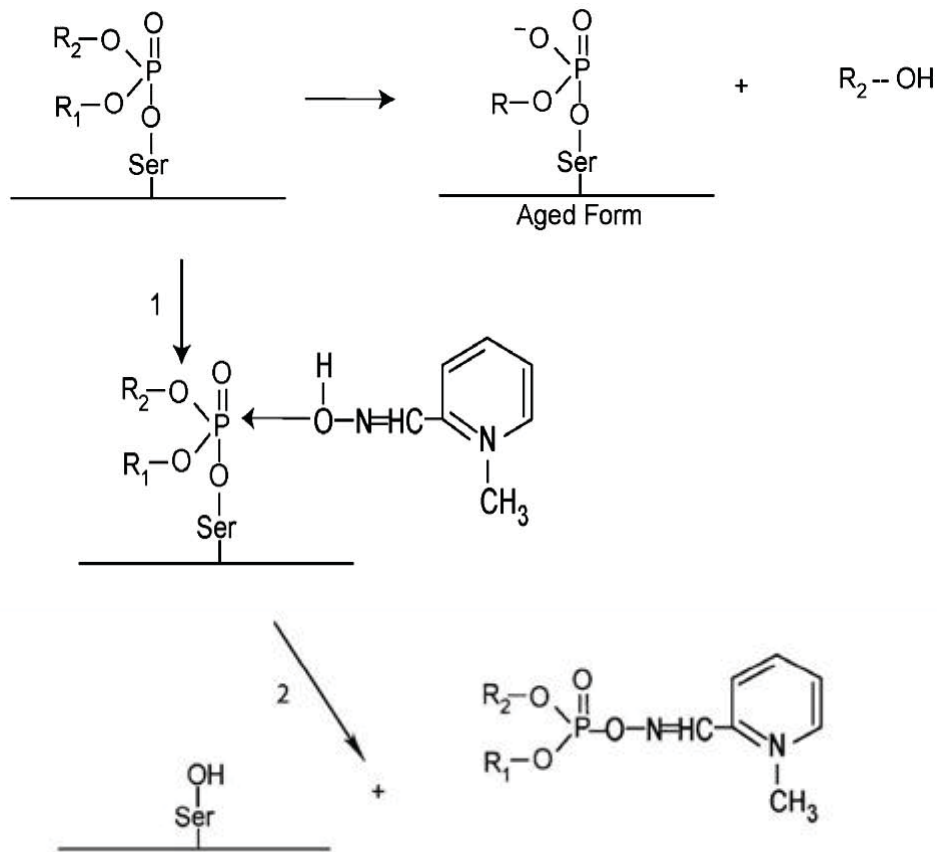
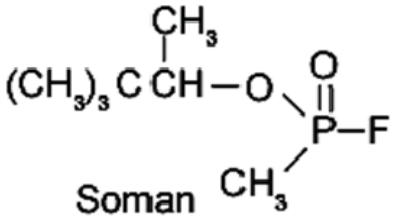
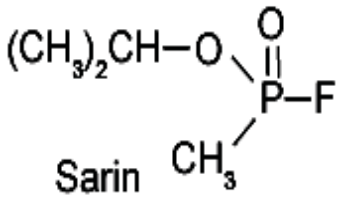


Figure 3 Mechanism of OP "aging" and reactivation by 2-PAM

The nerve agent inhibits AChE by binding to the serine. 2-PAM reactivates the inhibited AChE by nucleophillic attack of the OP compound bound to the enzyme.



Pinacolyl methylphosphonofluoridate



Isopropyl methylphosphonofluoridate

Figure 4 Structures of two organophosphate nerve agents, soman and sarin.

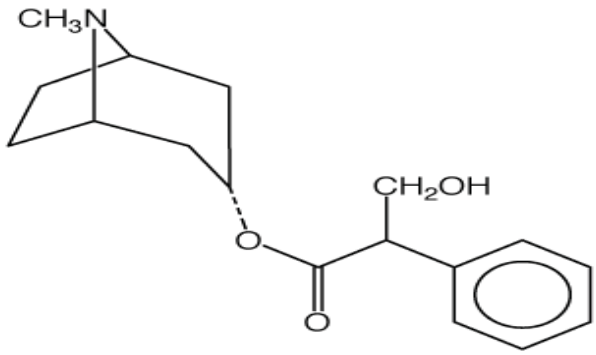


Figure 5 Structure of anticholinergic, atropine

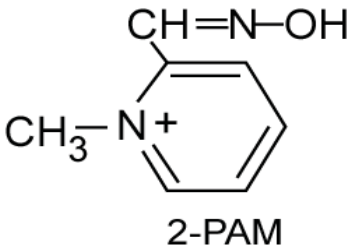


Figure 6 Structure of Pralidoxime (2-PAM)

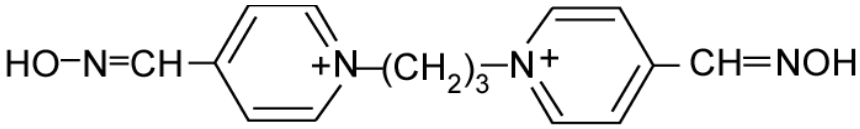
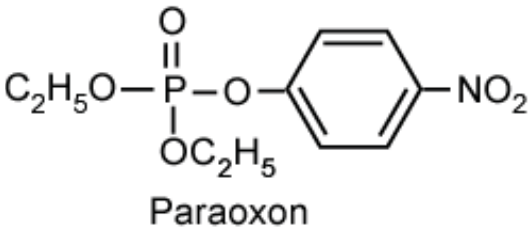
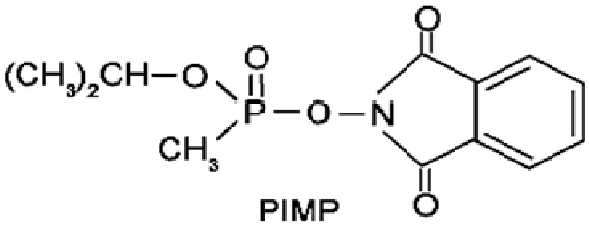


Figure 7 Structure of 1,1' Trimethylene bis(4-formylpyridiniumbromide) dioxime (TMB-4)



Paraoxon

Figure 8 Structure of organophosphate, paraoxon.



PIMP

Figure 9 Structure of sarin surrogate, phthalimidyl isopropyl methylphosphonate (PIMP)

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

The sarin surrogate, phthalimidyl isopropyl methylphosphonate (PIMP), paraoxon and all novel oximes were synthesized and generously supplied by Dr. Howard Chambers (Department of Entomology and Plant Pathology, Mississippi State University). 2-PAM was also provided by Dr. Howard Chambers.

2.2 Tissue Preparation for *in vitro* assays

Brains for *in vitro* analysis were obtained from Sprague-Dawley derived 250-300g male rats. Whole brain was homogenized at a concentration of 40 mg/ml in 0.05M Tris-HCl buffer (pH 7.4 at 37°C) using a Wheaton tissue grinder. 1.2 ml of homogenate were placed in individual tubes and stored in a -80°C freezer; this preparation is stable for up to a year

2.3 Percent Reactivation Determination

The determination of reactivation consisted of two phases, an inhibition phase and a reactivation phase. In the first phase (inhibition), rat brain was incubated with paraoxon or PIMP (designed to yield ~80% inhibition), and in the second phase (reactivation) inhibited AChE was incubated with an oxime.

The first phase consists of four individual reaction tubes; tubes one and three contain 10µl of ethanol as the OP vehicle. In tubes two and four, 10µl of paraoxon dissolved in ethanol (0.1µM final concentration) or the sarin surrogate PIMP $10^{-4.75}$M dissolved in ethanol (175nM final concentration) was added. The tubes were incubated with the appropriate tissue amount of 1ml for 15 minutes.

In the second phase, 10µl of 50%DMSO:50% EtOH was added to tubes one and two, and 10µl of a 10mM oxime dissolved in 50%DMSO:50% EtOH was added to tubes three and four resulting in a final oxime concentration of 100µM. The discontinuous acetylcholinesterase assay followed.

2.4 Discontinuous Acetylcholinesterase Assay

Acetylcholinesterase activity was measured spectrophotometrically using a modification (Chambers *et al*., 1988) of the widely used Ellman method (Ellman *et al*., 1961). Into four test tubes, 9.6 ml of buffer (0.05M Tris-HCl buffer, pH 7.4) and 0.4 ml of homogenate from the four individual test tubes described above in the percent reactivation determination assay were vortexed (total volume of 10ml). This was done to obtain the proper dilution for the AChE assay. From this dilution four sets of four tubes per set with 2 ml of the diluted homogenate were used for the discontinuous AChE assay.

The first tube of each set contained 20µl of eserine sulfate (10µM final concentration), a specific inhibitor of AChE, and served as a blank. All tubes were vortexed and placed in a water bath for 15 minutes at 37°C. Following the 15 minute incubation, 20µl of acetylthiocholine (ATCh) (1mM final concentration) was added as the substrate and the tubes were vortexed and returned to the water bath to incubate for 15 minutes. The reaction was stopped by the addition of 500 µl of a 4:1 solution of 5%

sodium dodecyl sulfate (SDS) and 0.024 M (5,5'-dithio-bis(nitrobenzoic acid)) (DTNB). Each tubes contents were poured into a cuvette and the absorbance was read at 412 nm using a Milton Roy Spectronic 1001 plus.

2.5 Percent Reactivation Calculation

The overall procedure consisted of two phases. Phase 1 was inhibition, where AChE in brain homogenate was incubated with paraoxon or PIMP. This phase was labeled as inhibition of A in the calculation. Phase 2 was reactivation, where inhibited AChE was incubated with an oxime. This phase was labeled as inhibition of B in the calculation. Inhibition and reactivation reactions were compared to appropriate controls to calculate percent inhibition and percent reactivation. Percent reactivation was calculated as follows: $[(\%I_A - \%I_B)/\%I_A] \times 100$ (I = Inhibition). Percent inhibition of A was determined by calculating $[(T_1 - T_2)/T_1] \times 100$ (T = tube). Tube one was the vehicle control which contains EtOH (OP vehicle) + 50% DMSO: 50% EtOH (oxime vehicle). Tube two was the oxime vehicle (organophosphate + vehicle). Percent inhibition of B was determined by calculating $[(T_3 - T_4)/T_3] \times 100$ (T = tube). Tube three was the vehicle (ethanol + oxime), and tube four organophosphate plus the oxime.

2.6 Partition Coefficients

Lipophilicity, reflecting the potential for crossing the blood-brain barrier, was measured by determining partition coefficients. N-Octanol/water partition coefficients were determined. Oxime was dissolved in distilled water and the solution was shaken vigorously with an equal volume of alcohol. The alcohol phase was dried over anhydrous

sodium sulfate, and the oxime concentration was determined by measuring the UV absorbance.

2.7 Normality

SAS was used to determine whether the data were approximately normally distributed. This was done to see if the data set was well-modeled by a normal distribution.

2.8 Structure Activity Relationship

Four series of groups based on structural similarities were compared for structure-activity relationship. Oximes differ based on the size and substituents. The structures of the oximes are not shown for proprietary reasons.

2.9 Statistical Analysis

The results obtained from this study were statistically analyzed using Microsoft Office Excel 2007, Sigma Plot 8.0, and SAS® System for Windows, Version 9.1. The analysis was done to determine which oximes do not differ statistically in their reactivation and to compare the reactivation activity for further testing *in vivo*. An ANOVA test was performed on the data to determine whether the data were normally distributed using SAS®. At the level $p<0.05$ the General Linear Model (GLM) procedure was used to conclude a significant difference between 2-PAM and the novel oximes, and within ANOVA a mean separation of the oximes was done. Oximes were grouped by similarity in structure and relative potency in comparison to 2-PAM was determined using Microsoft Excel 2007.

CHAPTER III

RESULTS

Twenty-eight novel oximes were synthesized and tested for their abilities to reactivate rat brain AChE following its inhibition by PIMP *in vitro*. All the oximes tested demonstrated some ability to reactivate inhibited AChE. Reactivation varied among the novel oximes 24-78% (Table 1) and the novel oximes were not as effective as 2-PAM or TMB-4, which reactivated 91% and 97%, respectively. Compared to 2-PAM, the novel oximes displayed efficacy of 37% to 86%. The lipophilicity for all the oximes was greater than 2-PAM or TMB-4 by 3-to 374-fold.

Twenty-eight oximes were tested for their efficacy of reactivating inhibited AChE, and of those 28 oximes 12 were tested with paraoxon. When using SAS to compare whether or not the two inhibitors were significantly different, it was determined that the two inhibitors, PIMP and paraoxon, were not significantly different at the 0.05 level of significance ($Pr > F = 0.0716$). To test whether the effects of the two inhibitors were significantly different one-way ANOVA was used. The novel oximes were more effective in reactivating AChE inhibited by paraoxon, but there was not a substantial difference between the two (Figure 10). When looking at the comparison chart of the inhibitors, oximes 2, 3, 6, 11, and 15 seemed to be substantially different. For this reason a student t-test was run on the oximes. When running the student t-test 5 of the 12 comparisons were significantly different, which were oximes 2, 3, 6, 11, and 12 (table 2).

Rat brain AChE showed low to moderate reactivation by the oximes when inhibited by PIMP (Table 1). In table 1, values are expressed as mean ± standard deviation (SD) where n=3. Significant differences were determined using SAS, where normality was also determined. To test whether the novel oximes have the same effect as 2-PAM, t-tests were run to determine whether or not the oximes were significantly different from 2-PAM. It was determined to reject the null hypothesis that they are similar. 2-PAM reactivated inhibited AChE better than all the novel oximes. When running distribution analysis using SAS to test for normality the Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling tests were run in which the results were normally distributed. To better visualize this, a QQ plot was run to check for normality using the average of the means (figure 11).

To better visualize the reactivation potential of the novel oximes in comparison to 2-PAM, figures 12-15 compared the relative potency of all oximes to 2-PAM. Oximes were grouped by similarity in structure to evaluate the reactivation potential. 2-PAM is given the number of 1 (Oxime/2-PAM=Relative Potency). In figure 12, 2 of 5 oximes showed above average reactivation potency and varied from 0.41 to 0.86. Group 2 (figure 13) had the most oximes with relative potency in the average range from 0.26-0.79. Group 2 had 6 out of 14 oximes showing average reactivation in comparison to 2-PAM. Group 4 (figure 14) had only one oxime out of 5 showing above average reactivation, and group 5 (figure 15) only had two oximes showing an average reactivation in comparison to 2-PAM. Although 2-PAM reactivated AChE better than the novel oximes, it showed the lowest lipophilicity. Lipophilicity ranged from 0.009 to 2.244 (Table 1).

Table 1 Characterization of novel oximes *in vitro*

Oxime	% Rat Brain Reactivation*	Relative Reactivation	Lipophilicity**	Relative Lipophilicity
2-PAM	91±5	1.00	0.006	1.0
TMB-4	97±4	1.10	<0.001	0.2
Oxime 1	37±6	0.41	0.029	4.8
Oxime 2	37±5	0.41	0.020	3.3
Oxime 3	39±4	0.44	0.009	1.5
Oxime 4	65±5	0.71	0.138	23
Oxime 5	78±3	0.86	0.117	19.5
Oxime 6	41±1	0.45	0.084	14
Oxime 7	49±4	0.54	0.072	12
Oxime 8	53±4	0.58	0.017	2.8
Oxime 9	54±10	0.60	0.128	21.3
Oxime 10	47±3	0.52	0.051	8.5
Oxime 11	46±6	0.51	0.031	5.2
Oxime 12	34±2	0.37	0.018	3
Oxime 13	34±2	0.37	0.034	5.7
Oxime 14	65±8	0.71	0.184	30.7
Oxime 15	24±4	0.26	0.680	113.3
Oxime 16	43±1	0.47	0.045	7.5
Oxime 17	72±8	0.79	0.174	29
Oxime 18	72±5	0.79	0.172	28.7
Oxime 19	56±6	0.62	0.352	58.7
Oxime 20	30±6	0.33	0.042	7
Oxime 21	39±1	0.42	0.146	24.3
Oxime 22	38±6	0.42	0.031	5.2
Oxime 23	48±3	0.52	0.056	9.3
Oxime 24	71±7	0.78	0.318	53
Oxime 25	42±1	0.46	0.084	14
Oxime 26	48±1	0.51	0.045	7.5
Oxime 27	28±2	0.31	0.538	89.7
Oxime 28	25±4	0.27	2.244	374

* 15-min incubation with PIMP following 30-min incubation with 100μM oxime, n=3 replications, mean ± SD

** n- Octanol/water partition coefficient Relative reactivation and lipophilicity as compared to 2-PAM

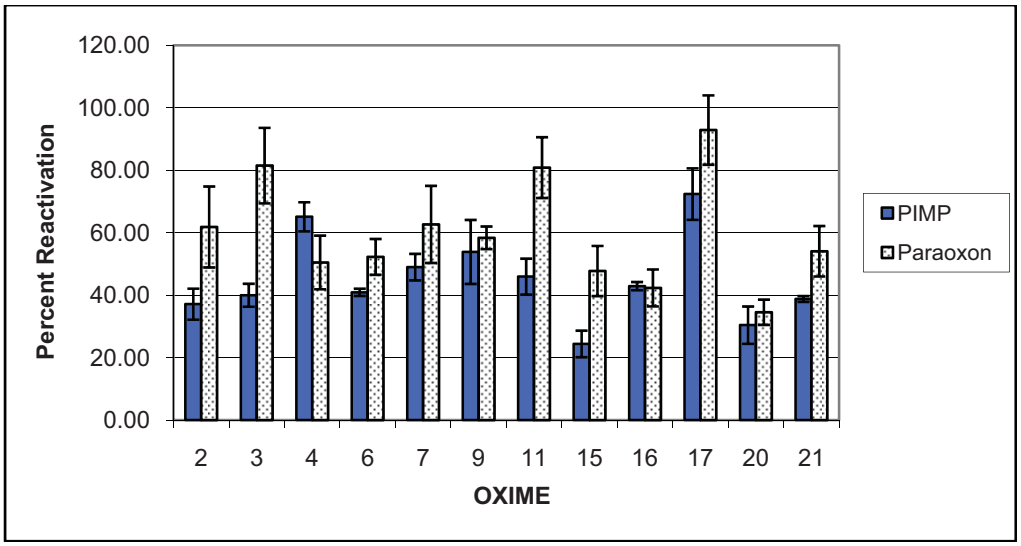


Figure 10 Mean comparison of PIMP and Paraoxon

Values are expressed as mean ± SD (n=3). Significant differences were determined using SAS where it was determined that the two inhibitors are not significantly different at the 0.05 level of significance.

Table 2 Student t-test of Paraoxon/PIMP comparison

Paraoxon	Rep 1	Rep 2	Rep 3	PIMP	Rep 1	Rep 2	Rep 3	P-Value
2	69.80	68.85	46.91	2	32.64	36.28	42.47	0.036674
3	73.18	75.96	95.38	3	41.33	42.79	35.83	0.004711
4	50.18	59.26	42.07	4	68.82	59.87	66.63	0.060984
6	46.05	53.39	57.38	6	39.95	42.20	40.59	0.028406
7	52.91	58.58	76.54	7	44.19	52.34	50.44	0.143505
9	55.16	57.69	62.29	9	52.01	64.92	44.68	0.533711
11	70.37	82.6	89.59	11	51.37	46.56	39.91	0.010409
15	42.69	43.57	57.01	15	27.55	26.06	19.56	0.020449
16	42.31	36.42	48.25	16	43.03	44.17	41.53	0.875702
17	82.00	92.46	104.20	17	72.69	80.48	63.98	0.062215
20	30.92	38.92	33.78	20	30.82	36.18	24.23	0.378422
21	47.64	63.14	51.45	21	39.86	38.24	38.28	0.079971

Comparison of three replications of individual oxime with paraoxon and PIMP
* Less than 0.05 for the p-value, there is a significant difference in the reactivation

Table 3 Reactivation activities of novel oximes *in vitro* when using paraoxon

Oxime	Rep 1	Rep 2	Rep 3	AVG	STD DEV
2	69.80	68.85	46.91	61.85	12.95
3	73.18	75.96	95.38	81.50	12.09
4	50.18	59.26	42.07	50.50	8.59
6	46.05	53.39	57.38	52.27	5.74
7	52.91	58.58	76.54	62.67	12.33
9	55.16	57.69	62.29	58.38	3.61
11	70.37	82.60	89.59	80.85	9.72
15	42.69	43.57	57.01	47.75	8.02
16	42.31	36.42	48.25	42.32	5.91
17	82.00	92.46	104.2	92.88	11.10
20	30.92	38.92	33.78	34.54	4.05
21	47.64	63.14	51.45	54.07	8.07

15-min incubation with paraoxon following 30-min incubation with 100μM oxime
Rep= Replications. 3 replications were run on each oxime to test for efficacy.

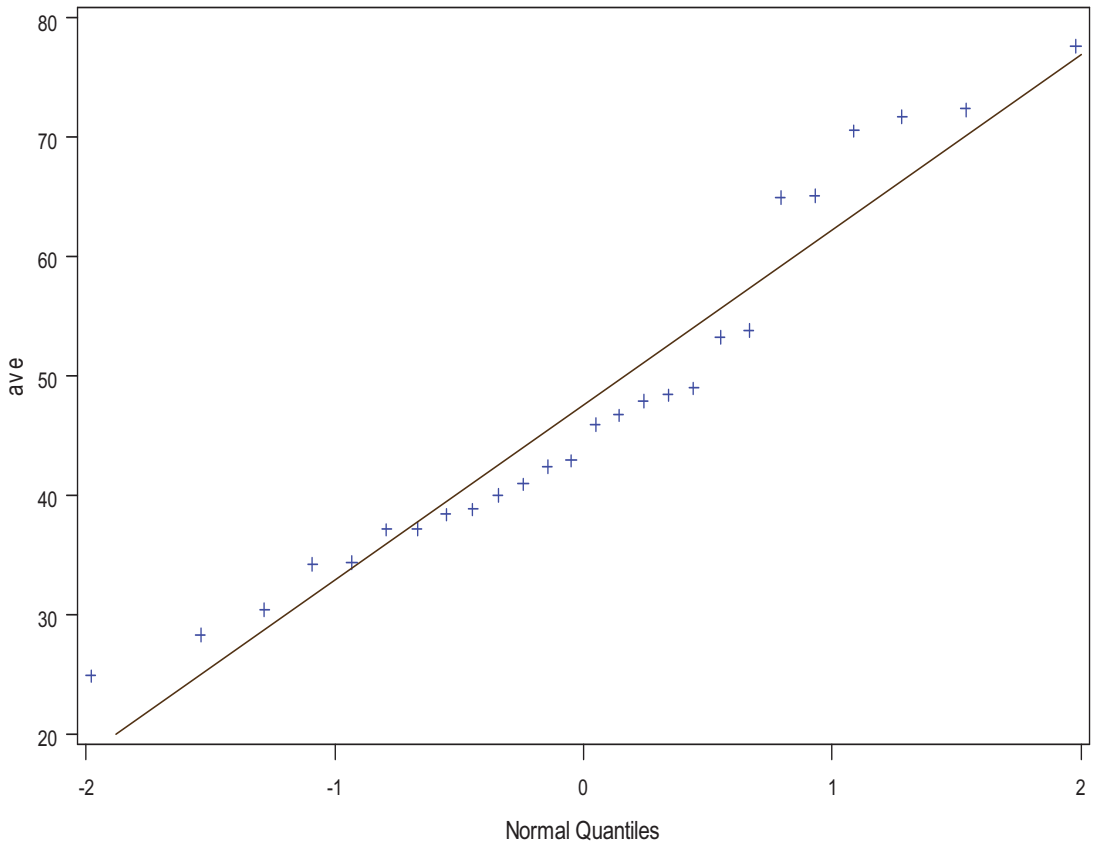


Figure 11 Normal probability test for normality testing

QQ plot showing that the assumption that the data are approximately normally distributed is acceptable.

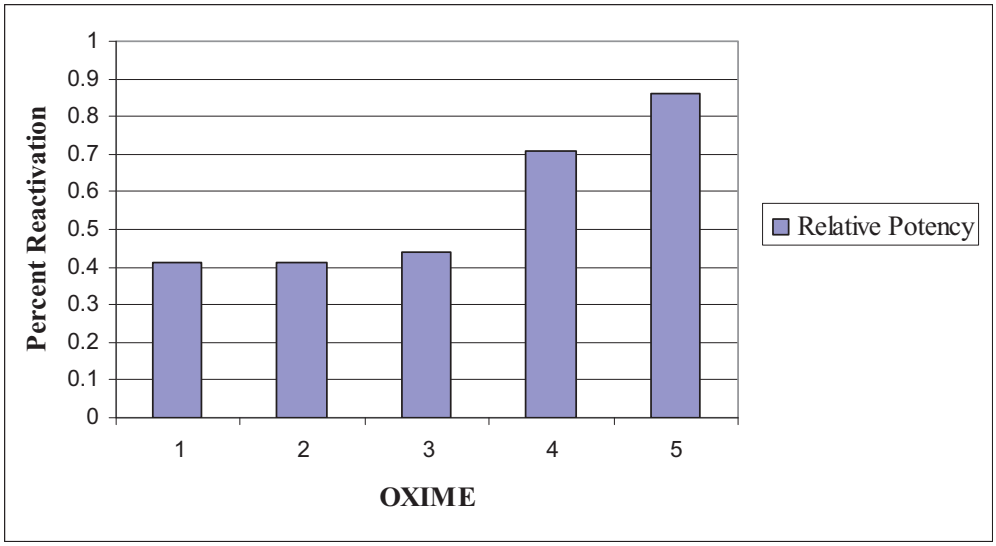


Figure 12 Chart showing relative potency of 5 oximes compared to 2-PAMranging from 0.41-0.86

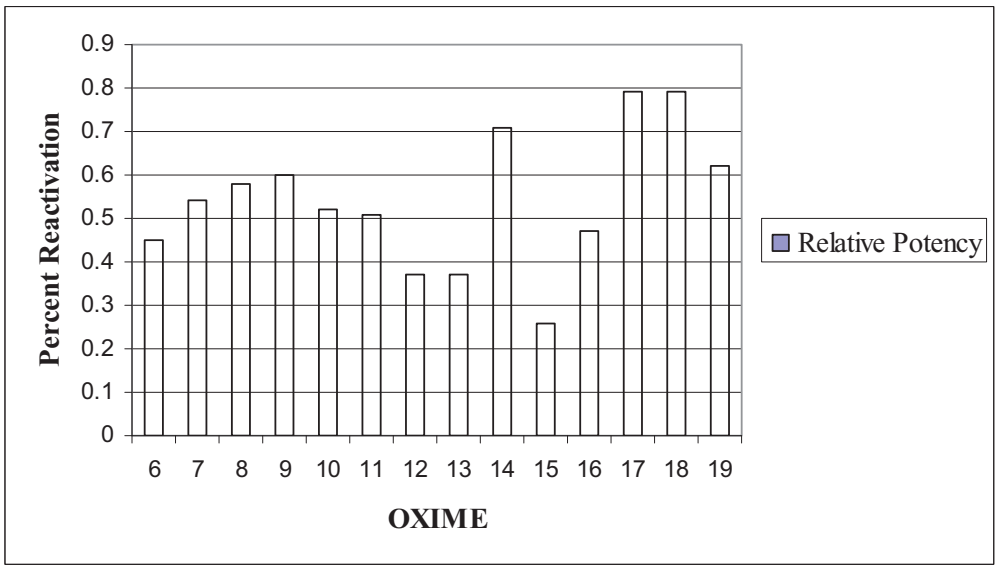


Figure 13 Chart showing relative potency of 14 oximes compared to 2-PAM ranging from 0.37-0.60.

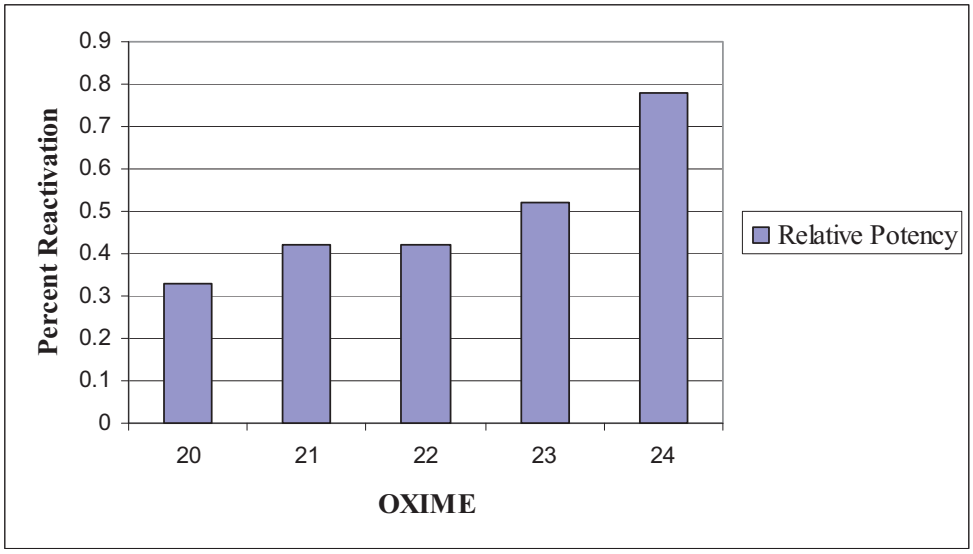


Figure 14 Chart showing relative potency of 5 oximes compared to 2-PAM ranging from 0.33-0.78.

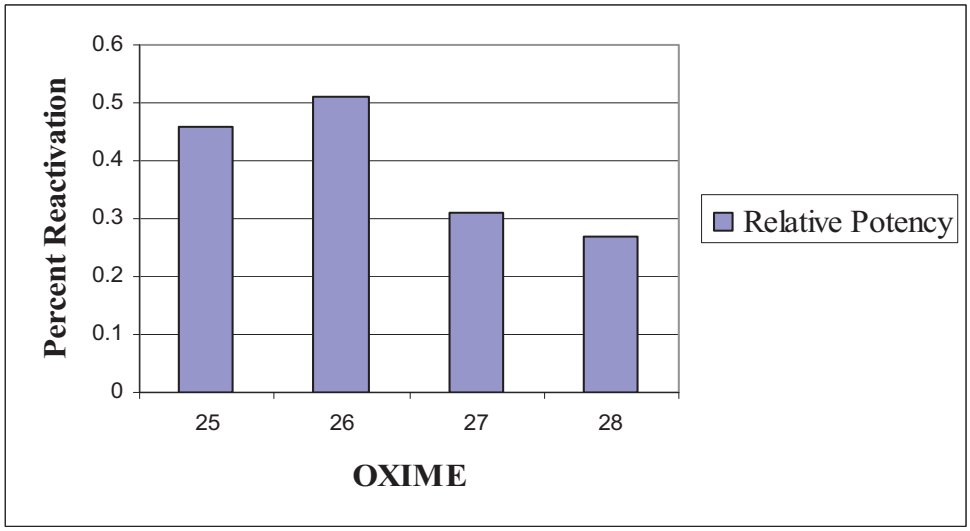


Figure 15 Chart showing relative potency of 4 oximes compared to 2-PAM ranging from 0.27-0.51

CHAPTER IV

DISCUSSION

The ability to quickly reactivate organophosphate inhibited AChE is extremely important in the event of nerve agent exposure. Current therapies for organophosphate poisoning (atropine and 2-PAM) are effective in reactivation of peripheral AChE, usually preventing death. However, 2-PAM and other existing oximes have very limited or no ability to cross the blood- brain barrier (BBB) and reactivate AChE. In this research, 28 novel oximes were designed and synthesized to be more lipophilic and the ability of these novel oximes to reactivate AChE were tested *in vitro*. While reactivation potencies were not as great as those of 2-PAM or TMB-4, there was appreciable reactivation showing that these oxime chemistries do have potential, and the lipophilicities have been greatly enhanced.

The function of the BBB is to protect the CNS from toxic molecules; this includes toxins ingested in food, inhaled, or absorbed through the skin. However the BBB also serves as a barrier to potentially beneficial drugs that will aid in the reactivation of inhibited AChE due to organophosphate poisoning (Haberman, 2009). The aim of this research was to determine whether a series of novel pyridinium oximes

synthesized to increase lipophilicity and thereby increase their likelihood of crossing the BBB, could effectively reactivate phosphorylated AChE *in vitro*. The results of this research demonstrate that it is possible to design an oxime that is potentially lipophilic enough (all novel oximes exceed the lipophilicity of 2-PAM) to penetrate the BBB and be effective in reactivating inhibited AChE in the CNS.

The role of an oxime is to remove the organophosphate moiety from the inhibited enzyme and restore the enzyme function. All oximes tested did not reach target reactivation efficacy(~50%). The mean reactivation rate of the oximes were significantly different when comparing the oximes to 2-PAM. This suggests that there are differences among the oximes in their ability to reactivate inhibited AChE. The ability of these oximes to reactivate AChE inhibited by phthalimidyl isopropyl methylphosphonate (PIMP) can depend on many things such as chemical structure of the inhibitor and reactivator, time of inhibition and reactivation, conditions (i.e., temperature, pH), and contamination of enzyme source (Kuca *et al*., 2007). For example, obidoxime is thought to be somewhat better that 2-PAM in treating victims with nerve agent poisoning, but obidoxime (a 4-position oxime) forms a stable phosphoryl oxime that inhibits reactivated AChE which may limit its use in the treatment of nerve agent poisoning. This explains the importance of why the chemical structure of the inhibitor and reactivator are important in oxime development. In tissue homogenates there are enzymes that are present which can interfere with the assay by reacting with substrates or products of assay reactions (Scheer *et al*., 2004). In this research the oxime/inhibitor is assayed directly in brain tissue homogenate under conditions in which the effect of possible contamination are minimal, and allows for more accurate results of the efficacy of the oximes.

The activities of the twenty-eight novel oximes are shown as relative activities compared to 2-PAM. 2-PAM is the current standard treatment for organophosphate poisoning in the United States. Current reactivators include different types of oximes with a similar basic structure differing by number of pyridinium rings and by the position of the oxime group in the pyridinium ring (Kuca *et al*., 2007). Structure-activity relationship is what helps to improve and synthesize new oximes. These twenty-eight novel oximes vary in their ability to reactivate inhibited AChE as well as in their structure. No single oxime is the same. Kuca *et al*. (2007) stated that the differences in the oxime efficacy toward nerve agents can be caused by oxime concentration in the target organs and various aging rate at which inhibited AChE is converted to a form that can no longer be reactivated by oximes. The actual oxime concentration that is present in the brain tissue homogenate may differ. Oxime concentration in peripheral target sites is practically the same as its concentration in the blood (Kassa, 2002; Bajgar, 2004; Worek *et al*., 2005). In *in vitro* analysis the exact determination of the reactivating potency of oximes requires the consideration of different reactions between AChE, OP, and oxime. Post-inhibitory reactions of AChE, aging, and spontaneous reactivation by oximes may affect the reactivation when testing *in vitro.* The therapeutic efficacy of a reactivator is dependent on its concentration in the brain tissue homogenate during incubation and on the reactivatability of inhibited AChE (Kuca *et al*., 2007).

A major factor in oxime penetration of the BBB is chemical structure. The polar ionic character of many oximes causes restriction by the blood-brain and the blood cerebrospinal fluid barriers. For this reason, the lipophilicity of an oxime is the simplest predictor of its potential to cross the BBB. This study's ultimate goal was to develop and

synthesize a phenoxyalkyl pyridinium oxime that was sufficiently lipophilic to penetrate the BBB, yet still be effective in reactivating AChE. Some oximes had considerably low reactivation rates, but possessed average lipophilicity. For example, oxime 28 had an average reactivation rate of 25% but its lipophilicity was very high at 2.244. Oxime 5, on the other hand was found to be the most effective reactivator but its lipophilicity was low at 0.117. It seems there is a median where limited reactivation and lipophilic potential can be effectively achieved. It was observed that lower reactivation rates seem to correlate with higher lipophilicity with some variability, which makes these oximes beneficial for testing *in vivo*.

When testing relative potency to 2-PAM, oximes 6-12 showed the least amount of range in reactivation. The reactivation ranged from 34-54% and lipophilicity from 0.018 and 0.128. Although this group of oximes did not vary in range of reactivation much, most of the oximes were below 50% and lipophilicity low. This suggest that due to the design of these particular oximes, potential for crossing the BBB and successfully reactivating inhibited AChE *in vivo* is not likely.

These differences can be due to many factors; as stated earlier an oxime's ability to reactivate AChE as well as penetrate the BBB has a lot to do with its chemical structure. It has been noted by many researchers that reactivation potency depends on structural factors, such as the number of pyridinium rings, the position of functional oxime groups, and the length of the bridge between two pyridinium rings (Jun *et al*., 2006; Ohta *et al*., 2006). Ohta *et al*. (2006) noted that when testing 40 hydrophobic PAM type oxime antidotes that were inhibited by a sarin analogue, 2-PAM showed the best recovery activity, but the activities of the PAM analogues were reduced by side chain

elongation. Berend *et al*. (2008) also investigated four new pyridinium derivatives with similar basic structure, but differed in length of the linker between two pyridinium rings and in the position of the oxime group in the pyridinium to determine their reactivating potency in human erythrocyte AChE, one of the four oximes showed a better reactivating potency than TMB-4. This was believed to be due to the position of the oxime group and the length between two pyridinium rings in the oxime structure (Berend *et al*., 2008). Although the novel oximes that were studied differ in their position of the oxime group on the pyridine ring, their reactivity is comparable because of the similarity in basic structure (Kassa, 2002).

The use of nerve agents is strictly controlled, and can be dangerous to inexperienced laboratory workers. PIMP is a considerably safer and non-volatile analogue of sarin that is conveniently prepared for testing of the novel oximes, and is substantially less hazardous to laboratory personnel. PIMP proved to be a good test agent that worked just as well in inhibiting AChE as paraoxon, and is considerably more relevant to nerve agent therapy. The phthalimidyl derivative degrades quickly in aqueous solution; this is particularly useful in *in vitro* assays of reactivation efficacy to prevent re-inhibition of reactivated AChE and therefore provide more accurate results. PIMP leaves AChE phosphorylated by the same chemical moiety as sarin and therefore provides a particularly useful chemical surrogate. In comparison to recent studies done by Ohta *et al*. (2006), who synthesized a non-toxic and stable sarin analogue (isopropyl *p*-nitrophenyl methylphosphate, INMP) to evaluate nerve agent antidotes; PIMP can be easily handled, it enabled rapid assay of reactivation activities of a large number of oximes, and provided rapid screening and evaluation of oximes.

Paraoxon and nerve agents are highly toxic OPs with high inhibition potency against AChE (Jun *et al*., 2008). Paraoxon proved to be a good inhibitor to test the potency of the novel oximes, and when comparing PIMP to paraoxon there is no significant difference between the two. In a recent study testing the potency of several oximes using paraoxon as the inhibitor for rat brain AChE, only obidoxime and trimedoxime reached sufficient reactivation efficacy, using the modified Ellman method for AChE activity analysis. Petroianu *et al*. (2006) have shown that, in rats, paraoxon induced mortality can be reduced by administration of oximes, but the protection conferred is insufficient considering that 30-90% of the animals died despite oxime treatment. These results lead to the conclusion that, whether inhibited by organophosphorus pesticides or nerve agents, there is no one oxime that is a good reactivator of poisoning from all types of OPs, and furthermore able to counteract CNS effects of organophosphates.

In vitro analysis is vital in the development of new AChE reactivators since the results can determine the most potent AChE reactivators for *in vivo* testing. *In vitro* testing can be used to determine the oxime's affinity toward intact and inhibited AChE (Kuca, 2005). Because *in vitro* studies always lack the complexity of *in vivo* systems, there is always the concern that *in vitro* data may not accurately reflect the oxime's potential *in vivo*, but many studies have proved this may not be the case. *In vitro* testing however does allow for the early exclusion of candidate oximes that reactivate AChE poorly, thereby reducing the amount of animal testing required. This is beneficial for both ethical and financial reasons. *In vitro* testing also allows the interaction of an oxime with the pharmacological target and in this particular research it would be the interaction with

AChE. Several studies have demonstrated that there is a correlation between *in vitro* and *in vivo* data, and the effectiveness of an oxime primarily depends on its ability to reactivate nerve agent inhibited AChE (Oldiges *et al*., 1970; Schoene *et al*., 1973). If the *in vitro* oxime reactivation of PIMP inhibited rat brain AChE reactivates in a similar way to that of human AChE, it is likely that the results of *in vivo* animal study will reliably extrapolate to humans (Luo *et al*., 2007).

In summary, the importance of reactivating OP-inhibited brain AChE should not be underestimated. Of all currently available oximes it is important to note that the pyridinium oximes appear to be the most effective at demonstrating AChE reactivation potential. Twenty-eight novel pyridinium oximes have been synthesized and tested for the reactivation potential. The data indicate that these oximes can reactivate inhibited AChE, and potentially cross the BBB. This furthermore proves that an oxime can be developed to show better BBB penetration than currently available oximes. It also worthy to note that these oximes in conjunction with 2-PAM can possibly be beneficial for survival of and effective recovery from nerve agent intoxication.

REFERENCES

687, Resolution. (n.d.). Adopted by the Security Council at its 2981st meeting on April 3, 1981.

Abdel-Rahmanl, A., Shetty, K., & Abou-Donia, M. (2002). Acute exposure to sarin increases blood brain barrier permeability and includes neuropathological changes in the rat brain: dose-response relationships. *Neuroscience* , 113:721-741.

Antonijevic, B., & Stojiljkovic, M. P. (2007). Unequal Efficacy of Pyridinium Oximes in Acute Organophosphate Poisoning. *Clinical Medicine and Research* , 5(1):71-82.

Baggot, J. (1994). Application of interspecies scaling to the bispyridinium oxime HI-6. *Am. J. Vet. Res.* , 55:689-691.

Bajgar, J. (2004). Organophosphate/nerve agent poisoning: mechanism of action, diagnosis, prophlaxis, and treatment. *Adv. Clin. Chem.* , 38:151-216.

Benke, G. a. (1975). The influence of age in the toxicity and metabolism of methyl parathion in male and female rats. *Toxicol. Appl. Pharmacol.* , 31, 254-269.

Benschop, H., & De Jong, L. (2001). Toxicokinetics of nerve agents. In S. Somani, & J. Romano, *Chemical Warfare Agents: Toxicity at Low Levels* (pp. 25-81). Boca Raton, Fl: CRC Press.

Berend, S., Vrdoljak, A., Radic, B., & Kuca, K. (2008). New bispyridinium oximes: In vitro and in vivo evaluation of their biological efficency in soman and tabun poisoning. *Chemico-Biological Interactions* , 175:413-416.

Burger, A., & Hobbiger, E. (1951). The inhibition of cholinesterases by alkylphosphates and alkylphenolphosphates. *Brit. J. Pharmacol.* , 6:593-605.

Chambers J.E., W. S. (1988). Effects of acute paraoxon and atropine exposures on retnetion of shuttle avoidance behavior on rats. *Neurosci Res Comm* , 3:85-92.

Clement, J., Bailey, D., Madill, H., Tran, L., & Spence, J. (1995). The acetylcholinesterase oxime reactivator HI-6 in man:pharmacokinetics and tolerability in combination with atropine. *Biopharm. Drug Dispos.* , 16:415-425.

Dawson, R. (1994). Review of oximes available for treatment of nerve agent poisoning. *J. Appl. Toxicology* , 14:317-331.

Ecobichon, D. (2001). Toxic Effects of Pesticides. In C. Klassen, *Casarett & Doull's Toxicolgy: The Basic Science of Poisons.* (pp. 779-787). New York, New York: McGraw-Hill.

Ecobichon, D. (1996). Toxic effects of pesticides. In M. D. Amdur, *Casarett & Doull's Toxicology: The basic science of poisons.* Newyork, NY.: McGraw-Hill.

Ellman, G. C. (1961). A new and rapid determination of acetylcholinesterase activity. *Biochem. Pharmacol.* , 7:88-93.

Fleischer, J., & Harris, L. (1965). Dealkylation as a mechanism for aging of cholinesterase after poisoning with pinacolyl mehylphosphonofluoridate. *Biochem. Pharmacol.* , 14:641-650.

Ge, S. S. (2005). Where is the Blood-Brain Barrier...Really? *Journal of Neuroscience Research* , 79:421-427.

Grob D, H. A. (1953). The effects and treatment of nerve gas poisoning. . *Am J Med* , 14:52-63.

Haberman, A.B. (2009). Strategies to overcome blood-brain barrier. *Genetic Engineering & Biotechnology,* 29:48-49.

Hackley, B., Steinberg, G., & Lamb, J. (1959). Formation of potent inhibitors of AChE by reactivation of pyridinaldoximes with isopropyl methylphosphonofluoridate (GB). *Arch. Biochem. Biophys.* , 80:211-214.

Hammond PI, K. C. (2003). Cholinesterase reactivation in vivo with a novel bis-oxime optimized by computer-aided design. *J. Pharmacol. Exp. Ther.* , 307:190–196.

Hobbiger, F. (1951). Inhibition of cholinesterases by irreversible inhibitors in vitro and in vivo. *Brit. J. Pharmacol.* , 6:21-30.

Johnson, J. a. (1987). Species-realted differences in the inhibition of brain acetylcholinesterase by paraoxon and malaoxon. *Toxicol. Appl. Pharmacol.* , 88:234-241.

Johnson, M., Jaconson, D., Meredith, T., Eyer, P., Heath, A., Ligtenstein, D., et al. (2000). Evaluation of antidotes for poisoning by organophosphorous pesticides. *Toxicology Series. Emergency Medicine* , 12:22-37.

Jun, D., Musilova, L., Kuca, K., Kassa, J., & Bajgar, J. (2008). Potency of several oximes to reactivate human acetylcholinesterase and butyrycholinesterase inhibited by paraoxon in vitro. *Chemico-Biological Interactions* , 175:421-424.

Kassa, J. (2002). Review of oximes in the antidotal treatment of posioning by organophosphorous nerve agents. *Journal of Toxicology-Clinical Toxicology* , 40(6):803-813.

Kuca K, P. J. (2003). Reactivation of organophosphate inhibited acetylcholinesterase activity by a,ω-bis(4-hydroxyiminomethylpyridinium) alkanes in vitro . *J. Appl. Biomed* , 1: 207–211.

Kuca, K. a. (2003). A comparison of the ability of a new bispyridinium oxime-1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)butane dibromide and currently used oximes to reactivate nerve agent-inhibited rat brain acetylcholinesterase by in vitro methods. *J. Enzyme Inhib. Med. Chem.* , 18:529–535.

Kuca, K., Cabal, J., Jun, D., & Musilek, K. (2007). In vitro reactivation potency of acetylcholinesterase reactivators-K075 and K075- to reactivate tabun-inhinbited human brain cholinesterases. *Neurotoxicity Research* , 11(2):101-106.

Kuca, K., Cabal, J., Kassa, J., Jun, D., & Hrabinová, M. (2005). Comparison of in vitro potency of oximes (pralidoxime,obidoxime, HI-6) to reactivate sarin-inhibited acetylcholinesterase in various parts of pig brain. *J. Appl. Toxicol.* , 25:271–276.

L.W., H., Fleischer, J., Clark, J., & Cliff, W. (1966). Dealkylation and loss of capacity for reactivation of cholinesterases inhibited be sarin. *Science* , 154:404-406.

Lamb J.C., S. G. (1966). Isopropyl methylphosphonylated bisquaternary oximes; powerful inhibitors of cholinesterase. *Biochem. Biophys. Acta* , 89:174–176.

Ligtenstein, D. (1986). On the synergism of the cholinesterase reactivating bispyridinium-aldoxime HI-6 and atropine in the treatment of organophosphate intoxications in the rat. . *MS thesis. Univ. of Leyden* .

Marrs, T. (2007). Toxicology of organophosphate nerve agents. In T. Marrs, R. Maynard, & F. Sidell, *Chemical Warfare Agents: Toxicology and Treatment 2nd Edition* (pp. 191-222). England: John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester.

McDonough, J., & Shih, T. (1995). A study of the N-Methyl-D-Aspartate antagonistic properties of anticholinergic drugs. *Phamacol Biochem and Behavior* , 51:249-259.

Misulis, K., Clinton, M., & Dettbarn, W.-D. (1987). Differnces in central and peripheral neural actions between soman and diisopropyl flurophosphate, organophosphorous inhibitors of acetylcholinesterases. . *Toxicol Appl Pharmacol* , 89:391-398.

Mortensen, S., Brimijon, S., Hooper, M., & Padilla, S. (1998). Comparsion of the in vitro sensitivity of rat acetylcholinesterase to chlorpyrifos-oxon: what do tissue IC50 values represent? *Tox. and Appl. Pharm.* , 148:46-49.

Nagao, M., Takatori, T., Matsuda, Y., NaKajma, M., Iwase, H., & Iwadate, K. (1997). Definitive evidence for the acute sarin poisoning diagnosis in the Tokyo subway. . *Toxicol. Appl. Pharmacol.* , 150:310-320.

Nair, V., & Hunter, J. (2004). Anticholinesterases and anticholinergic drugs. *Continuing education in anaesthesia, crtitical care & pain* , 4(5):165-168.

Nolte, H., Rosenberry, T., & Neuman, E. (1980). Effective charge on acetylcholinesterase active sites determined from the ionic strength dependence of association rate constants with cationic ligands. *Biochem.* , 19:3705-3711.

Ohta, H., Ohmori, T., Suzuki, S., Ikegaya, H., Sakurada, K., & Takehiko, T. (2006). New safe method for preparation of sarin-exposed human erythrocytes acetylcholinesterase using non-toxic and stable sarin analogue isopropyl p-nitrophenyl methylphosphonate and its application to evaluation of nerve agent antidotes. *Pharmaceutical Research* , 23:2827-2833.

Oldiges, H. a. (1970). Pyridinium and imidazolium salts as antidotes for soman and paraoxon poisoning in mice. *Arch Toxicol.* , 26:293-395.

Petroianu, G., Arafat, K., Kuca, K., & Kassa, J. (2006). Five oximes (K-27, K-33, K-48, BI-6 and methoxime) in comparsion with pralidoxime in vitro reactivation of red blood cell acetylcholinesterase inhibited by paraoxon. . *Appl Toxicol.* , 26(3):262-268.

Rang, H., Dale, M., & Ritter, J. (1999). Pharmacology. *Churchill Livingstone, 4th Edition* , 131-135.

Richardson, J. (MS Thesis, Mississippi State Univ, MS 69 pages). *Analysis of the in vitro inhibition of acetylcholinesterase by mixtures of chlorpyrifos-oxon and azinphos-methyl-oxon: are effects purely additive?*

Richter, J., & A., W. (1971). Evidence for separate systems for the transport of neutral and basic amino acids across the blood-brain-barrier. *Neurochem* , 18:613-620.

Sakurada, K., Matsubara, K., Shimizu, K., Shiono, H., Seto, Y., Tsuge, K., et al. (2003). Pralidoxime iodide (2-PAM) penetrates across the blood-brain barrier. *Neurochem. Res.* , 28(9):1401-1407.

Scheer, W., Lehman, H., & Beeler, M. (1978). An improved assay for hexokinase activity in human tissue homogenates. *Analytical Biochemistry* , 91:451-463.

Schinkel, A. (1999). P-Glycoprotein, a gatekeeper in the blood-brain barrier. *Advanced Drug Delivery Reviews* , 36:179-194.

Schoene, K. a. (1973). Efficacy of pyridinium salts in tabun and sarin poisoning in vivo and in vitro. *Arch. Int. Pharmacodyn. Ther.* , 204:110-123.

Sellstrom, A., Algers, G., & Karlson, B. (1985). Soman intoxication and the blood-brain barrier. *Fundam. Appl. Toxicol.* , 5:122-126.

Shih, T., Duniho, S., & McDonough, J. (2003). Control of nerve agent-induced seizures is critical for neuroprotection and survival. *Tox and Appl Pharmacol* , 188(2):69-80.

Soreq, H., & Zakut, H. (1993). *Human cholinesterases and anticholinesterases.* San Diego, CA: Academic Press, INC.

Tsuji, A. (1998). Strategies for drug delivery to the brain across the blood-brain barrier. *Nippon Rinsho* , 56:613-618.

Weapons, S. A. (2005). *Nerve Agents.* http://www.opcw.org/resp/html/nerve.html.

Wilson, B., Hooper, M., Hansen, M., & Nieberg, P. (1992). Reactivation of organophosphorous inhibited acetylcholinesterase with oximes. In E. b. Levi, *Organophosphates: Chemistry, Fate, and Effects.* San Diego, CA: Academic Press.

Worek, F., Szinicz, L., Eyer, P., & Thiermann, H. (2005). Evaluation of oxime efficacy on nerve agent poisoning: development og a kinetic based dynamic model. *Toxicol. Appl. Pharmacol.* , 209:193-202.

Worek, F., Thiermann, H., Szinicz, L., & Eyer, P. (2004). Kinetic analysis of interactions between human acetylcholinesterase, structurally different organophosphorous compunds and oximes. . *Biochem Pharmacol* , 68:2237-2248.

APPENDIX A

DUNNETT TEST

When comparing the oximes to 2-PAM, 28 different hypothesis test were run. For each test there is a 5% chance of making a type I error to reject the null hypothesis when you are not supposed to but, overall there is a much larger chance of making at least one Type I error over all 28 tests. This probability is 1-(1-.05)^28= .7622. Thus having a 76% chance of making at least one type I error when conducting 28 tests. To make this probability smaller, the Dunnett test is used that guarantees that the probability that you are making at least one Type I error is .05, instead .7365 if no correction is used. The Dunnett's test compared the group means (n=3) to a reference group (2-PAM) where the difference between means where determined at a 95% confidence limits for LSMean (i) – LSMean (j) where (i) is the oxime and (j) is the three replications ran for each oxime.