Molecular, Biochemical, and Toxicological Evaluation of Anticholinesterases for control of the Malaria Mosquito, *Anopheles gambiae*

James Mutuku Mutunga

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Entomology

Jeffrey R. Bloomquist

Kevin M. Myles

Sally L. Paulson

Igor V. Sharakhov

Troy D. Anderson

April 28, 2011
Blacksburg, Virginia

Key words: *N*-methylcarbamates, active site gorge, tacrine dimers, selectivity, Acetylcholinesterase, oximes, pyrazoles, Bivalent carbamates, Akron strain

Molecular, Biochemical, and Toxicological Evaluation of Anticholinesterases for control of the Malaria Mosquito, *Anopheles gambiae*

James Mutuku Mutunga

ABSTRACT

Pyrethroids are the only class of insecticides approved by the World Health Organization (WHO) for use in insecticide treated nets (ITNs), the first line of malaria vector control. Widespread resistance development to pyrethroids undermines current control efforts, and hence an urgent need for alternative chemistries. I report the evaluation of pharmacological differences between insect and vertebrate acetylcholinesterase (AChE) as well as selectivity and toxicity testing of new carbamate insecticides on *Anopheles gambiae*, the African malaria mosquito. AChE gorge pharmacology data revealed differences between insect and vertebrate AChE that can be exploited in the design of a bivalent insecticide. Toxicokinetic analysis showed that metabolic detoxication and cuticular penetration affect toxicity of carbamates in a manner dependent on the chemical structure.

Structure activity relationships of side-chain branched *N*-methylcarbamates emphasized the importance of structural complementarity of ligands to the AChE catalytic active site and the substrate, acetylcholine. Monovalent pyrazoles and acetophenone oxime carbamates were toxic to both susceptible and carbamate-resistant mosquitoes carrying a G119S mutation within the catalytic site. A bivalent phthalimide-pyrazole carbamate and sulfenylated phenyl *N*-methyl carbamates were highly toxic when topically applied onto insect but less toxic by treated filter paper assays. *In vitro* evaluation of a molecular mosquito-selectivity model using AChE peripheral site ligands confirmed that selectivity of PRC 472 was due to presence of I70 in mosquito, which is Y70 in human AChE. The

findings presented here are important steps in the on-going search of a mosquito-selective

and resistance mitigating carbamate insecticide for control of malaria mosquitoes.

Dedication

To my dear loving wife Kathambi, and our children Mwende and Mutunga

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

x

LIST OF TABLES

Chapter 1. Literature Review

1.1 Mosquito control in the management of vector borne diseases

Mosquitoes are vectors of a number of diseases such as malaria, dengue fever, the more severe dengue hemorrhagic fever (DHF), yellow fever, eastern equine encephalitis, and filariasis (Krogstad, 1996; Roberts, 1997; Gubler and Clark, 1995). Malaria is the world's most important parasitic infection and a major health and developmental challenge. Despite years of continual efforts to eliminate malaria, this disease is still one of the major causes of morbidity and mortality affecting third-world countries. The World Health Organization (WHO) estimates that in 2008 malaria caused 190 - 311 million clinical cases, with 0.8 to 1 million deaths; 89% of which occur in Africa (WHO 2008). In 1992, the WHO set up sustainable strategies against malaria disease, focusing on the proper treatment of malaria cases and the use of preventive measures against the vectors (WHO, 2002b). Vector control remains the most generally effective measure to prevent malaria transmission and is therefore among the basic technical elements of the Global Malaria Control Strategy (WHO, 1992).

The principal objective of vector control is the reduction of malaria morbidity and mortality by reducing the levels of transmission although control methods vary considerably in their applicability, cost and sustainability of their results (Bay, 1967). It is certainly true that vector control does not require total elimination of the vector in order to control vector borne diseases. For instance, in North America and Europe, *Anopheles* mosquitoes capable of transmitting malaria are still present, but the parasite has been eliminated (CDC, 2004). Socio-economic improvements (e.g., houses with screened windows, air conditioning) combined with vector reduction efforts and effective treatments have led to the elimination of malaria without the need for complete eradication of the vectors (Lindsay *et al.,* 2002).

Several methods are being used for mosquito control and their success depends on a variety of factors. Before the discovery of dichloro-diphenyl-trichloroethane (DDT), the main approach to controlling anopheline vectors was directed at the larval stage, which required a detailed knowledge of the bionomics of local vectors (Brogdon and McAllister, 1998). Larvicidal approaches are still used for mosquito control with varying levels of success (Mittal, 2003; Brogdon and McAllister, 1998). Only in projects of high economical and political value was a highly disciplined organization enforcing the application of antilarval measures able to achieve spectacular success notably, the eradication of invading populations of *Anopheles gambiae* from Brazil and Egypt, but not *An. darlingi* in Brazil which breeds in rivers (Shousha, 1948). Current control measures involve the use of a variety of methods such as synthetic chemicals, larvicides, environmental management, and community participation.

Chemical insecticides are used in a variety of ways that include fogs, indoor residual sprays (IRS) and insecticide treated nets (ITNs), among others. In Africa, the use of IRS and ITNs are currently the principle methods approved by WHO for mosquito control (WHO, 1998). Several studies have reported the reduced malaria morbidity and mortality, associated with use of ITNs (ter Kuile *et al.,* 2003; Armstrong-Schellenberg *et al.* 2001; Lengeler 1998; Curtis *et al.,* 1998; Choi *et al.* 1995; D'Alessandro *et al.,* 1995). Evidence of success in controlling mosquitoes using insecticides is faced with the threat of failure from widespread insecticide resistance (Brogdon and McAllister, 1998). Sustainable use of insecticides in mosquito control therefore requires not only new insecticides, but also a new generation of insecticides that are more effective and selective, less harmful to humans, and unlikely to select for insecticide resistance, all of which are major goals of our research.

1.2 Mechanisms of anticholinesterase resistance

The major type of anticholinesterase resistance is biochemical, and can be classified into target site insensitivity (e.g. G119S mutation of *ace*-1 gene in mosquitoes) and metabolic resistance (Brogdon and McAllister, 1998). Target site insensitivity has been reviewed extensively in insects (Ranson *et al.*, 2004; Brogdon and McAllister, 1998; Fournier and Mutero, 1994). Target site insensitivity in insects is associated with several different mutations in *ace*-1 and *ace*-2 genes, which code for AChE-1 and AChE-2, respectively. Some insects have both genes but only the *ace*-1 is functionally important in neurotransmission (Bourguet *et al.,* 1996; Weill *et al.,* 2003). Mutations involving *ace*-2 have only been reported in three dipteran species namely, *Drosophila melanogaster*, *Musca domestica* and *Bactrocera oleae* (Mutero *et al.,* 1994; Menozzi *et al.,* 2004; Kozaki *et al.,* 2001; Vontas *et al.,* 2002). Point mutations in the *ace*-2 gene have been described in insecticide resistant strains of *D. melanogaster* (Mutero *et al.,* 1994; Fournier *et al.,* 1992). Mutations in the *ace*-2 gene of *M. domestica* and *B. oleae* have been described (Walsh *et al.,* 2001; Vontas *et al.,* 2002). Studies in Central American and Mexican populations of *An. albimanus* have shown that an altered AChE is the most common OP/ CB resistance mechanism (Penilla *et al.*, 1998, Ayad and Georghiou, 1979; Hemingway and Georghiou, 1983). AChE target-site insensitivity has been reported from *Culex pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, An. nigerimus, An. atroparvus, An. sacharovi* and *An. gambiae* (Bisset *et al.,* 1990; N'Guessan *et al.,* 2003). A G119S mutation in the *ace*-1 gene has been identified in target site insensitive AChE strains of *Cx. pipiens, Cx. quinquefasciatus* and *An. gambiae* (Weill *et al.,* 2002; Weill *et al.,* 2003).

Common metabolic resistance mechanisms in insects are modified levels or activities of esterase detoxification enzymes that metabolize insecticides (Brogdon and McAllister, 1998). Esterases are a family of proteins that hydrolyze ester linkages (Cygler *et al*, 1993; Oakeshott *et al*, 1993). A single amino acid change converts the specificity of an esterase to an insecticide hydrolase or multiple-gene copies maybe amplified in resistant insects (e.g. the B1 and A2-B2 amplicons in *Cx. pipiens* and *Cx. quinquefasciatus*) (Mouches *et al*, 1990; Vaughan *et al*, 1997).

Cytochrome P450 monooxygenases metabolize insecticides through *O*-, *S*-, and *N*-alkyl hydroxylation, aliphatic or aromatic hydroxylation and epoxidation, ester oxidation, and nitrogen or thioether oxidation (Wilkinson, 1976). A NADPH linked reaction mediates these modifications. Epoxidation of a cyclohexene results in 1,2 cyclohexanediol that is easily excreted. Dipteran P450 monooxygenases occur as a cluster of genes (Maitra *et al*, 1996), and resistance results from constitutive overexpression rather than amplification (Carino *et al*, 1994). Thus, several genes copies maybe present in resistant insects. Elevated levels of P450 monooxygenases have been implicated in insecticide resistance in mosquitoes, often in conjunction with other mechanisms. For example, Djouaka *et al* (2008) reported elevated levels of P450s in *An. gambiae* populations resistant to multiple pyrethroids. The Akron strain of *An. gambiae* possess both a G119S mutation in the *ace*-1 gene, and the L1014F mutation associated with *kdr*. *Ace*-1 gene duplication has been reported in *An. gambiae*, whereby the second mutation has less fitness cost than the first, and therefore likely to remain fixed in a population (Djogbénou *et al*, 2009). Cross-resistance may also occur whereby resistance to one class of insecticides leads to resistance to another class, especially if their targets are related (e.g. *kdr* or oxidases for cross-resistance to DDT and pyrethroids) (Brengues et al., 2003; Brogdon et al., 1999).

Organisms may express different classes of multiple glutathione-*S*-transferases (GSTs) (Hayes *et al.*, 1995; Zhou and Syvanen, 1997) confering resistance to different insecticides. For example, in *Ae. aegypti,* resistance to DDT is associated with a cluster of GST genes (Grant *et al*, 1991). These clusters can exist in multiple forms, even in the same insect (Prapanthadara *et al*, 1996; Ranson *et al*, 1997). GSTs primarily function either directly through dehalogenation or dealkylation, or by catalyzing the secondary metabolism of a vast array of compounds first oxidized by cytochrome P450 family (Ranson and Hemingway, 2004). GSTs also play an important role in the physiology stress and have been implicated in intracellular transport and various biosynthetic pathways (Wilce and Parker, 1994). GSTs metabolize OPs via *O*-dealkylation or *O*-dearylation (Oppernoorth *et al.*, 1979). In *O*-dealkylation, glutathione (GSH) is conjugated to the alkyl portion of the insecticide, whereas reaction of GSH with the leaving group is *O*-dearylation (Chiang and Sun, 1993). Since OP insecticides are typically non-insecticidal, GSTs also catalyze their secondary metabolism *in vivo*. For example, the phosphorothionate form of OPs is activated to the insecticidal oxon form by P450s in *An. subpictus*, whereas GSTs facilitate conjugation of the toxic oxon to non-toxic analogs by hydroxylation (Hemingway *et al.*, 1991). Rodriguez *et al* (2002) showed that temephos resistant *Ae. aegypti* were also resistant to organophosphates and pyrethroids due to elevated levels of GSTs. Multiple resistance mechanisms may occur due to sequential exposure of different classes of insecticides (Georghiou, 1994)

Synergists are a broad class of chemicals that serve as alternative substrates for metabolic enzymes and therefore reduce the rate of insecticide detoxification *in vivo*. They are considered to be non-toxic but potentiate the toxicity of insecticides by acting as alternate substrates or reacting with the active site to affect covalent inhibition. An example of the latter is *O*-ethyl *O*-

p-nitrophenyl phenylphosphonothioate (EPN), a poor AChE inhibitor but it undergoes oxidative

desulfuration *in vivo* (Metcalf, 1967), to yield a synergist. The level of synergism is assessed by

determining the LD_{50} or LC_{50} of toxicant divided by that of toxicant with synergist. Synergists

are used in mixtures to control insect pests and disease vectors. They enhance the effects of

insecticides by reducing metabolic sequestration of the toxicophores (Review by Metcalf, 1967

and references therein). Mixing sesamex or piperonyl butoxide with pyrethroids increases the

efficacy and reduces the amount of active ingredient required, and reduces cost. Synergists have

also been used to restore activity of chemicals in which resistance has developed due to elevated

monooxygenases and carboxyesterases. For example, 1,1 *bis p*-chlorophenyl-ethanol (DMC) was

used as synergist for DDT-resistant flies (Hennesay, 1961). Synergists lower the resistance ratio

of insect populations to chemical insecticides. The diverse chemical structures of synergists

restore the sensitivity of diverse enzyme targets. However, not all synergists are synthetic

chemicals. Studies with the synergist sesame oil showed that activity was dependent on 3, 4

methylenedioxyphenol moieties (Haller *et al*. 1942), and the effect was higher in alkyl or

alkenyl, ester, ether, amide, sulfone and sulfoxide benzene-substituted analogs (Beroza and

Marthel (1957).

1.3 Insecticide resistance in *An. gambiae* in Kenya

Insecticide resistance threatens the reemergence of vector borne diseases by affecting disease

control efforts (Krogstad, 1996; WHO, 1992). Resistance to organophosphates (OPs) was first

reported 14 years after their introduction and has since been documented in 260 insect and mite

species. Carbamate (CB) insecticide resistance appeared after five years, partly conditioned by

previous OP exposure (Hirshhorn, 1993). With the prospect of field-testing new carbamate

insecticides, it is important to establish the resistance status of mosquito populations, in areas earmarked for evaluation of field-efficacy. In Asembo-bay, initial reports estimated the frequency of the pyrethroid-resistant *kdr* allele at 3% (Ranson *et al.,* 2000). Four years later, frequencies were estimated at 11% (Stump *et al.,* 2004), an approximately 4-fold increase. *Kdr* frequencies for mosquitoes sampled in Mbita, Mwea, and Kwale were 8%, 6% and 13%, respectively (Mutunga *et al.,* 2005). Elevated P450 monooxygenases to permethrin was reported in five western villages, after a five-year period of ITN use (Vulule *et al.,* 1999). To date, there is no documented evidence of carbamate or OP resistance in mosquito populations in Kenya, despite recent countrywide mapping of pyrethroid resistance (Chen *et al*., 2008; Ranson *et al.* 2010). Unlike pyrethroid resistance, which occurs at low levels even without selection, the G119S mutation of *ace*-1 is associated with high fitness cost (Weill *et al.* 2004; Djogbénou *et al.* 2008; Newcomb *et al.* 1997). It is therefore unlikely to occur without selection pressure. Although pyrethroid exposure to mosquitoes may result from IRS and ITNs, exposure to carbamates and organophosphates likely occurs through the use of agricultural pesticides, as was observed with DDT and carbosulfan in West Africa (Akogbeto *et al.* 2005, N'guessan *et al.* 2003).

1.4 Organophosphates and carbamates as anticholinesterases

Organophosphate (OP) and carbamate (CB) anticholinesterases (Fig. 1.1) have been used to control mosquitoes and other insects for over half a century, establishing a long track record of efficacy as insecticides (O'Brien, 1967). Unfortunately, anticholinesterase insecticides are toxic to insects and humans thereby limiting their use in vector control.

Figure 1. 1. Examples of covalent and non-covalent acetylcholinesterase inhibitors.

The insecticidal effects of carbamates and organophosphates are attributed to their ability to inhibit acetylcholinesterase (AChE). The carbamylated or phosphorylated enzyme is no longer capable of hydrolyzing acetylcholine, which results in the buildup of the neurotransmitter (ACh) at the nerve synapse (Cohen and Oosterbaan, 1963). Accumulation of ACh causes excessive excitation of the nerve cells leading to hyperactivity, uncoordinated movements, tremors, convulsions and paralysis (Colhoun, 1958; Idris *et al.,* 1986; Booth and Metcalf, 1970). At postganglionic muscarinic synapses, ACh accumulation leads to parasympathetic activity of smooth muscle in the lungs, gastrointestinal tract, heart, eyes, bladder, secreatory glands, and increased activity in the postganglionic sympathetic receptors for sweat glands (Kiss and Fazekas, 1979). Excessive ACh at nicotinic motor end plates causes persistent depolarization of skeletal muscle (analogous to that of succinylcholine), resulting in fasciculations, progressive

weakness, and hypotonicity (Worek *et al.,* 2005). Moreover, as OPs cross the blood-brain barrier, they may cause seizures, respiratory depression, and CNS depression (Worek *et al.,* 2005; Leblanc *et al.,* 1986). OPs and CBs also bind to erythrocyte and plasma esterases, causing minimal clinical effects, which are useful in confirmatory diagnostic studies (Thiermann *et al.,* 1999).

Carbamates are *N*-substituted esters of carbamic acid that can be classified into an aryl group (*e.g.*, propoxur and carbofuran, Fig. 1.1) or an aliphatic group (*e.g.*, aldicarb and methomyl, Fig. 1.1) (Coppage, 1977). Currently, the WHO Pesticide Evaluation Scheme (WHOPES)-recommends carbamate insecticides bendiocarb WP (0.1-0.4g/m^2) and propoxur WP (1-2g/m^2) for residual spraying against malaria vectors. These carbamates have an effective action duration of 2-6 months (WHO, 2002a). The EPA has withdrawn all carbamates from household use following the enactment of the Food Quality Protection Act (EPA cumulative risk assessment report, 2007).

OPs are generally toxic; however, active ingredients within the group possess varying degrees of toxicity and consequently have been divided into several classes based on their LD_{50} values in rats (Minton and Murray, 1988). Class I is most toxic (e.g. chlorfenvinphos) and has an LD_{50} in the range 1-30 mg/kg. The oral LD_{50} range for class II (e.g. dichlorvos), is 30-50 mg/kg, while the third and least toxic class (e.g. malathion and diazinon) has a range of 1,000-10,000 mg/kg and 130-1,000 mg/kg; respectively depending on the protein content of the rat diet (Gallo and Lawryk, 1991; NLM, 1992). WHOPES-recommended organophosphate insecticides for residual spraying against malaria vectors are fenitrothion WP (2g/m^2), malathion WP (2g/m^2) and pirimiphos-methyl WP (1-2g/m2), with an effective duration of action of 2-6 months (WHO, 2002a).

Due to the current problems associated with insecticides available for vector control, there is need for new chemicals to sustain the important role of insecticides in public health.

1.5 Structural biology of acetylcholinesterase

A three-dimensional (3-D) structure of AChE from the electric eel, *Torpedo californica* (*Tc*AChE) has provided valuable insights in to structure-function relationships (Sussman *et al.,* 1991). High-resolution crystal structures of multiple AChE proteins, with and without bound ligands, are also now available for molecular modeling efforts (Bourne *et al.,* 2004; Bartolucci *et al.,* 2001). These structures have proven useful in ligand docking and for the synthesis of anticholinesterase compound libraries. Additionally, availability of the *An. gambiae* genome (Weill *et al.,* 2002) provides an opportunity to exploit gene sequence homology analysis in the design of anticholinesterases.

The AChE catalytic subunit is comprised of a peripheral site that opens to the external milieu, and a catalytic anionic site (CAS) at the bottom of a 20Å deep narrow gorge (Fig. 1.2). The peripheral anionic site (PAS) is located at the mouth of the gorge. The bottleneck has been shown to exclude large ligands from accessing the CAS, and some mutations at this site are implicated in insecticide resistance. The esteratic site sits at the bottom of the gorge, and binds an acetyl group of the substrate, while the oxyanion hole stabilizes the tetrahedral intermediate, during ACh hydrolysis (Fig. 1.2). A G119S mutation renders the enzyme resistant to carbamylation and is associated with ca. 30% loss in catalytic efficiency possibly due to an unstable tetrahedral intermediate (Lenormand *et al.* 1999). The anionic site binds a quaternary ammonium of ACh and other ligands through π-cation or π-π interactions (Ma *et al.,* 1997). The acyl pocket binds an acetyl moiety of ACh.
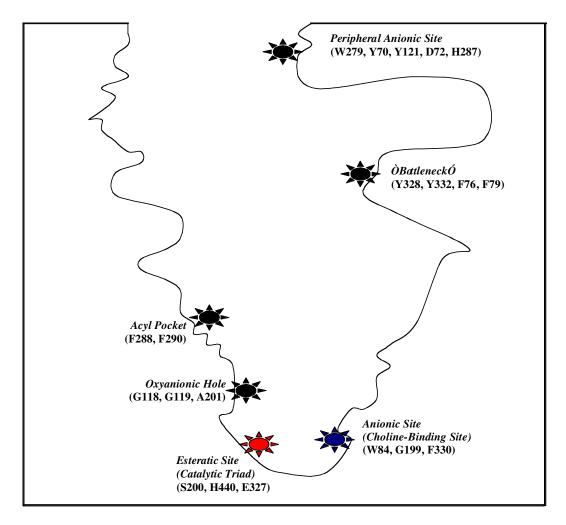
Figure 1. 2. Depiction of AChE gorge, showing key amino acid residue sites. For consistency, the numbering in figure 1.2 and the text is based on *T. californica* AChE (Harel et al. 1993, Sussman et al. 1991), unless stated otherwise.

Fourteen conserved aromatic residues (~ 40%) line the AChE gorge, providing multiple hydrophobic binding sites (Sussman *et al.* 1991; Savini *et al.* 2003; Toda *et al.* 2003). Recent reports have shown that the cysteine at position 286 of *Ag*AChE peripheral site is absent in humans (Pang, 2006). Homology models have also shown differences near the peripheral and active sites of *Ag*AChE and *h*AChE, notably I70/Y72, A81/T83, Y333/G342, M348/P446, D441/Y449, (*Ag*AChE/*h*AChE single letter code numbering; Hosea *et al.*, 1996; Pang, 2006; Mutunga *et al.,* unpublished). Previous studies have shown that the narrowest part of the gorge;

11

the bottleneck, is comprised of the aromatic residues Y121, F330 and F331 (Shen *et al.,* 2002; Botti *et al.,* 1999). The bottleneck plays an important role in preventing bulky ligands from accessing the catalytic site of AChE (Harel *et al.*, 2000; Felder *et al.*, 2002). More so, a F331W mutation is associated with organophosphate and carbamate insensitivity in *Culex tritaeniorhynchus,* the Japanese encephalitis vector mosquito and the spider mite, *Tetranychus kanzawai* (Nabeshima *et al.,* 2004; Aiki *et al.,* 2005). Specifically, the F353W mutation (*Aphioxus* sequence numbering) has a role in inhibiting the channeling of inhibitors to the catalytic triad of the CAS by constricting their passage and creating a 'bottleneck' in *amphioxus* AChE (Patel *et al.,* 2006).

1.6 Random and rational strategies for discovery of leads in insecticide design

While random screening programs have been used for decades in the discovery of a majority of pesticides (Tomlin, 2000), more rational approaches have been called for in pesticide design (Goosey, 1992; Menn and Henrick, 1981; Pillmoor *et al.,* 1991). An example of rational approach is Structure-Based Design (SBD) where the crystal structure of a biologically important enzyme or protein with its natural ligand or a bound inhibitor acts as a starting point in pesticide design (Magnus, 2002). On the basis of a protein-ligand structure, rational changes can be made to the structure of the natural ligand (or inhibitor) and their effect on its binding assessed (Edwards *et al.*, 1999). Re-designed compounds are then tested as inhibitors, and structures with the new inhibitors bound to the enzyme are obtained. This paradigm involves an iterative process of design, testing, and structure re-evaluation, hopefully leading to new molecules with very high binding affinity (Magnus, 2002; Winkler and Holan, 1992). Recent developments in molecular and structural biology are paramount to the discovery of leads showing *in vivo*

activity. *In vitro* and *in vivo* tests require large numbers of compounds and produce relatively few hits (Eliseev and Lehn, 1999). Classical lead discovery uses both rational and random design to generate compounds for screening. Compounds for random screening can come from historic compound collections, combinatorial chemistry, natural products, or often imitative approaches such as analog development from existing commercial products, a so called "me-too-chemistry" (Smith, 2002).

Rational design of compounds for screening refers to methods which preselect compounds on the basis of information about the target site of action (*i.e.*, SBD), or on the basis of structural similarity to known active compounds (Dolle, 1992). This latter approach encompasses the combinatorial exploration of leads designed on the basis of structural data (Gless *et al.,* 2001). SBD is a resource-intensive approach, requiring relatively large quantities of purified protein, access to X-ray facilities, and appropriate computational and modeling tools (Winkler and Holan*,* 1992). While SBD is commonly used by medicinal chemists in the design of new pharmaceuticals, there are far fewer examples of its application in the search for new pesticides designed for agricultural and public health use (Magnus, 2002; Doucet-Personeni *et al.,* 2001). Other methods of rational drug design include 3D-QSAR studies, model- or toxophore-based design and biochemically inspired design methods (Evans and Lawson, 1991).

A wealth of structural information for AChE makes it an ideal candidate for SBD. The best characterized is *Torpedo californica* (*Tc*) AChE, which serves as a model for all AChEs. It has a narrow 20Å narrow gorge with peripheral and catalytic sites (PAS, CAS) at the extremities of its active site gorge and is lined by aromatic residues forming an aromatic patch important in catalysis (Bergamann *et al.*, 1950; Botti *et al.,* 1999; Sussman *et al.*, 1999; Silman and Sussman, 2005). The well characterized structure of AChE combined with the availability of X-ray

13

crystallographic data provide an unprecedented opportunity for detailed study of structure-function relationships. The 14 conserved aromatic residues lining the gorge, represent about 70% of the total surface of the gorge (Axelsen *et al.,* 1994). These residues may represent appropriate sites around which to design specific insecticides. For example, AChE inhibition relieves the symptoms of Alzheimer's disease (AD) (Soreq and Seidman, 2001), and interactions between aromatic AChE residues and the quaternary ammonium ion of the natural substrate or its replacement in the inhibitor are crucial for potent binding of inhibitors (Ma and Dougherty, 1997). Additionally, differences in the second order amino acids surrounding the gorge may differ in their interactions with the conserved residues of insects and mammals, thereby providing unexpected possibilities for selective chemistry.

Doucet-Personeni *et al.* (2001) described an SBD approach to reversible AChE inhibitors with a view to the design of selective insecticides. These inhibitors were intended to combine the binding features of two previously known classes of AChE inhibitors, tacrine (THA) and trifluoromethylketone (TFMK) (Fig. 1.3). THA and TFMK are nanomolar inhibitors of AChE. Enzyme-inhibitor complexes show that THA forms stacking interactions with the aromatic residues F330 and Y84 (Fig. 1.3a), while TFMK binds to a different region of the active site (Fig. 1.3b). TFMK forms a covalent bond with S200 in the active site which acts as a mimic of the hemiketal transition state. This design of reversible inhibitors was based on the assumption that hybrid inhibitors bridging the tacrine binding site and the active site serine would have potent inhibitory properties (Doucet-Personeni *et al.,* 2001).

A

B
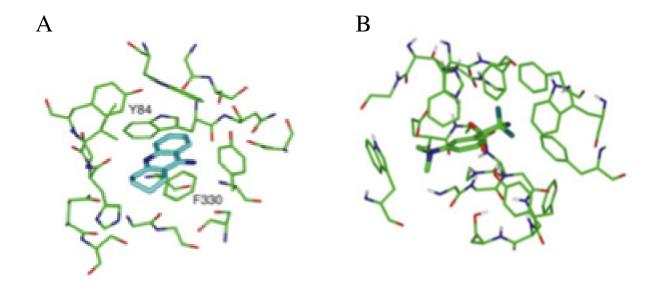
Figure 1. 3. (**A**) Tacrine (THA) bound in the active site of AChE. Carbon atoms of TA are coloured in cyan (Reproduced with permission; Doucet-Personeni *et al.,* 2001). (**B**) Trifluoromethyl ketone bound in the active site of AChE, TFMK carbon atoms are coloured in green (Reproduced with permission; Doucet-Personeni *et al.,* 2001).

1.7 Bivalent ligand strategy in the design of acetylcholinesterase inhibitors

Previous work has shown enhanced AChE affinity for bifunctional inhibitors such as the *bis*-quaternary inhibitors, decamethonium (DECA) (Bergmann *et al.,* 1950), BW284c51 (Austin and Berry, 1953) and ambenonium (Lands *et al.,* 1958; Du and Carlier, 2004), as well as tacrine dimers (Fig. 1.4), which are comprised of two tacrine moieties linked by a hydrocarbon spacer (Rydberg *et al.*, 2006). The catalytic and peripheral sites bind to the bivalent ligands simultaneously, giving rise to the "chelate effect", which is a well-known thermodynamic phenomenon. This is manifested as enhanced stability of the complex formed from bi- or polyvalent ligands and their targets, when compared to a similar complex formed from the monovalent counterparts (Haviv *et al.*, 2007). The crystal structure of AChE complexes bound to bivalent gorge-spanning ligands; DECA and BW284c51, explain the 'chelate effect' exhibited

by dual-binding inhibitors (Haviv *et al.*, 2007; Felder *et al.*, 2002). These structural assignments have been supported by extensive kinetic studies based on site directed mutagenesis (Taylor and Radic, 1994).

The availability of ligand-bound AChE crystal structure libraries are valuable reference structures in the *in silico* design and synthesis of candidate compounds. Chemical libraries can be generated through the application of multiple techniques, such as molecular docking, *in situ* click chemistry and new inorganic synthesis to generate new bivalent inhibitors. Bivalency, which has been shown to provide dramatic enhancements in inhibition potency and selectivity (Jencks, 1981), has been a very effective strategy for AChE involving tacrine and phenanthridinium components (Lewis *et al.,* 2002).
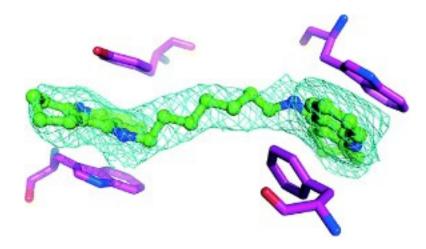
Figure 1. 4. Docking of a bivalent tacrine anticholinesterase (7 carbon tether) with *Tc*AChE (Reproduced with permission; Rydberg *et al.*, 2006).

1.8 Objectives of the study

This dissertation describes four research objectives, whose brief descriptions and justifications are provided below:

I.   The first objective was to use tacrine dimers as structural probes in mapping the gorge geometry of *Blattella germanica* and *Drosophila melanogater* AChE. These two insects were used as models to assess differences between AChE-1 and AChE-2, in comparison with other available insect and vertebrate gorge geometry data. This study provided important information on not only structural AChE differences across various insects and vertebrate AChEs, but also crucial factors to be considered in the design of bivalent insecticides. Details of this study are presented in Chapter 2.

II.   The toxicokinetics of newly discovered highly selective *N*-methylcarbamates in *Anopheles gambiae* mosquitoes were assessed. Specifically, I dissected the role of metabolic detoxification *in vivo*, and also assessed the role of the cuticle as a penetration barrier. Additionally, we tested the selectivity and toxicity of new pyrazol and oxime carbamates on *Anopheles gambiae* susceptible (G3) and carbamate resistant (Akron) mosquitoes. Conventional commercial *N*-methylcarbamates do not kill mosquitoes expressing the resistance *ace*-1 (G119S) phenotype. These oxime and pyrazole carbamates were designed to be effective against both susceptible and carbamate resistant mosquitoes. Details of toxicokinetic assays and efficacy of new resistance breaking carbamate insecticides are presented in Chapter 3.

III.   The effects of various structural modifications made to some of the new selective alkyl and silyl *N*-methylcarbamates (described in chapter 3), were assessed. We purposed to conduct these modifications in order to scale up for selectivity and toxicity, an often daunting task in

insecticide discovery. Specifically, I assessed effects thioalkyl analogs, side-chain branching and halogenated analogs of parental carbamates, described in detail in Chapter 4.

IV.   Several mutations at key residues of the mosquito enzyme to mimic the human enzyme were accessed to test several structural models and functional hypotheses. Recombinant mutants were then probed with ligands to assess the effect of those changes on catalytic efficiency, substrate specificity and potency to specific inhibitors and details presented in Chapter 5. At the AChE 'bottleneck' we made F329S, in order to examine the hypothesis that access of C286 to sulfhydryl reagents in AgAChE is blocked in space by F329. Therefore, the F329S mutant may not only mimic the HuAChE but also confer sensitivity to bulkier active site inhibitors and catalysis of bulky substrates. A new model generated by M. Totrov *et al.* (unpublished) suggested that the specificity of PRC 472, a benzylpiperidinyl derivative, is due to unique and specific interactions with I70 in *Ag*AChE. This residue is Y70 in HuAChE and therefore PRC 472 should be not potent to I70Y, if the model holds true. We also tested the potency of other PSLs known to be non-selective to AgAChE. Lastly, a double mutant of the mosquito enzyme was made, containing M438P and D441Y, to assess their effects on the selectivity observed with PRC 421 and related class 2 experimental carbamates.

References

Abbot WS, (1925). A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18: 265–267.

Akogbeto M, Djouaka R. and Noukpo H (2005) Use of agricultural insecticides in Benin, *Bull. Soc. Pathol. Exot*. 98: 400–405.

Aiki Y, Kozaki T, Mizuno H and Kono, Y (2005). Amino acid substitution in Ace paralogous acetylcholinesterase accompanied by organophosphate resistance in the spider mite Tetranychus kanzawai. *Pestic. Biochem. Physiol*. 82: 154–161.

Armstrong-Schellenberg JRM, Abdulla S and Nathan R (2001). Effect of large-scale social marketing of insecticide-treated nets on child survival in rural Tanzania. *Lancet* 357: 1241–1247.

Austin L and Berry WK (1953). Two selective inhibitors of cholinesterase. *Biochem. J.* 54: 695-700.

Ausubel FM, Brent R, Klingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (1987). *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience, NY.

Axelsen PH, Harel M, Silman I and Sussman, JL (1994). Structure and dynamics of the active site gorge of acetylcholinesterase: synergistic use of molecular dynamics simulation and X-ray crystallography. *Protein Sci*. 3: 188-197.

Ayad H and Georghiou GP (1979). Resistance pattern of *Anopheles albimanus* Wied, following selection by parathion. *Mosq. News* 39: 121-125.

Bartolucci C, Perola E, Pilger C, Fels G, Lamba D (2001). Three-dimensional structure of a complex of galanthamine (Nivalin) with acetylcholinesterase from *Torpedo carlifornica*: Implications for the design of new anti-Alzheimer drugs. *Proteins* 42(2): 182-191.

Bay EC (1967). Mosquito control by fish: A present-day appraisal. *WHO Chron*. 21: 415-423.

Bergmann F, Wilson, IB and Nachmansohn, D (1950). The inhibitory effect of stilbamidine, curare and related compounds and its relationship to the active groups of acetylcholine esterase. Action of stilbamidine upon nerve impulse conduction. *Biochim. Biophys. Acta* 6: 217-224.

Beroza M, and Barthel WF (1957). Chemical structure and activity of pyrethrin and allethrin synergists for control of housefly. *J. Agri. Food Chem.* 5: 855-859.

Binka F, Kubaje A, Adjuik M, Williams L, Lengeler C, Maude G, Armah G, Kajihara B, Adiamah J and Smith P (1996). Impact of permethrin impregnated bednets on child mortality in Kassena-Nankana district, Ghana: a randomised controlled trial. *Trop. Med. and Intern. Health*. 1: 47-54.

Bisset J, Rodriguez M, Soca A, Pasteur N and Raymond M (1987). Cross-resistance to pyrethroid and organophosphorus insecticides in the southern house mosquito (Diptera: Culicidae) from Cuba, *J. Med. Ent*. 41: 408–413.

Booth GM and Metcalf RL (1970). Histochemical evidence for localized inhibition of cholinesterase in the housefly. *Ann. Ent. Soc. Am*. 63: 197–204

Botti S, Felder CE, Lifson S, Sussman JL and Silman I (1999). Amodular treatment of molecular traffic through the active site of cholinesterase. *Biophys. J.* 77: 2430–2450.

Bourguet D, Raymond M, Fourier D, Malcolm CA, Toutant J-P and Arpagaus M (1996). Existence of two acetylcholinesterases in the mosquito culex pipiens (Diptera: Culicidae). *J. Neurochem*. 67: 2115-2123.

Bourne Y, Kolb HC, Radic Z, Sharpless KB, Taylor P and Marchot P (2004). Freeze-frame inhibitor captures acetylcholinesterase in a unique conformation. *Proc. Natl. Acad. Sci*. USA 101: 1449-1454.

Brogdon WG and McAllister JC (1998). Insecticide resistance and vector control. *Emerg Inf. Dis*. 4: 4-10.

Brown AWA (1986). Insecticide resistance in mosquitoes: a pragmatic review. *J. Americ. Mosq. Cont. Assoc*. 2: 123-140.

Carino FA, Koener JF, Plapp FW Jr, Feyereisen R (1994). Constitutive overexpression of the cytochrome P450 gene Cyp6A1 in a house fly strain with metabolic resistance to insecticides. *Insect Biochem Mol Biol*. 24: 411-8.

CDC (2004). Annual malaria report, National Center for Infectious Diseases, Division of Parasitic Diseases.

Chandre F, Darriet F, Manguin S, Brengues C, Carnevale P and Guillet P (1999). Pyrethroid cross-resistance spectrum among populations of Anopheles gambiae s.s. from Cote D'Ivoire. *J. American Mosquito Control Asso*. 15: 53–59.

Chen H, Githeko AK, Githure JI, Mutunga JM, Zhou G, Yan G (2008). Monooxygenase levels and knockdown resistance (kdr) allele frequencies in *Anopheles gambiae* and *Anopheles arabiensis* in Kenya. *J. Med. Entomol*. 45(2): 242-250.

Chiang F, and Sun C. (1993) Glutathione transferase isoenzymes of DBM larvae and their role in the degradation of some OP insecticides. Pestic. Biochem. Physiol. 45: 7-14.

Choi HW, Breman JG, Teutsch SM, Liu S, Hightower AW & Sexton JD (1995). The effectiveness of insecticide-impregnated bed nets in reducing cases of malaria infection: a meta-analysis of published results. *Am. J. Trop. Med. and Hyg*. 52: 377–382.

Coetzee M, Horne DWK, Brooke, BD and Hunt RH (1999). DDT, dieldrin and pyrethroid insecticide resistance in African malaria vector mosquitoes: an historical review and implications for future malaria control in southern Africa. *South Afri. J. of Science* 95: 215-218.

Cohen JA and Oosterbaan RA (1963). The active site of acetylcholinesterase and related esterases and its reactivity toward substrates and inhibitors. *Handbuch der Experimentellen Pharmakologie* 15: 300-373.

Colhoun EH (1958). Acetylcholine in Periplaneta Americana L.-II Acetylcholine levels in nervous activity. *J. of Insect Physiol.* 2(2): 117-120.

Curtis CF, Maxwell CA, Finch RJ & Njunwa KJ (1998). A comparison of use of a pyrethroid either for house spraying or for bednet treatment against malaria vectors. *Trop. Med. Intern. Heath.* 3: 619–631.

Cygler M, Schrag JD, Sussman JL, Harel M, Silman I, Gentry MK, (1993). Relationship between sequence conservation and three-dimensional structure in a large family of esterases, lipases and related proteins. *Protein Sci* 2: 366-82.

D'Alessandro U, Olaleye BO, McGuire W, Langerock P, Bennett S, Aikins MK, Thomson MC, Cham MK, Cham BA, Greenwood BM (1995). Mortality and morbidity from malaria in Gambian children after introduction of an impregnated bednet programme. *Lancet* 345: 479-483.

Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J, and Strode C (2008) Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of Anopheles gambiae s.s. from Southern Benin and Nigeria. *BMC Genomics*. 9: 538.

Dolle RE (1992). Comprehensive survey of combinatorial libraries with undisclosed biological activity. *Mol Diversity* 4: 233-256.

Doucet-Personeni C, Bentley PD, Fletcher RJ, Kinkaid A, Kryger G, Pirard B, Taylor A, Taylor R, Taylor J, Viner R, Silman I, Sussman JL, Greenblatt HM, Lewis T (2001). A structure-based design approach to the development of novel, reversible AChE inhibitors. *J Med Chem* 44(20): 3203-3215.

Dougherty DA (1996). Cation-pi interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. *Science*. 271: 163–168.

Du W, Awolola TS, Howell P, Koekemoer LL, Brooke BD, Benedict MQ, Coetzee M and Zheng L (2005). Independent mutations in the Rdl locus confer dieldrin resistance to *Anopheles gambiae* and *Anopheles arabiensis*. *Insect Mol. Biol*. 14 (2): 179-183.

Djogbénou L, Chandre F, Berthomieu A, Dabiré R, Koffi A, Alout H, Weill M (2008). Evidence of introgression of the ace-1R mutation and of the ace-1 duplication in West African *Anopheles gambiae* s.s. *Plos ONE.* 3(5):e2172.

Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J, and Strode C (2008) Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of Anopheles gambiae s.s. from Southern Benin and Nigeria. *BMC Genomics*. 9: 538.

Du DM, Carlier PR (2004). Development of bivalent acetylcholinesterase inhibitors as potential therapeutic drugs for Alzheimer's disease. *Curr. Pharm. Des*. 10: 3141-3156.

Edwards PJ, Gardner M, Klute W, Smith GF and Terrett NK (1999). Applications of combinatorial chemistry to drug design and development. *Curr. Opin. Drug. Disc. Develop*. 2: 321-331.

Eliseev AV and Lehn JM (1999). Dynamic combinatorial chemistry: Evolutionary formation and screening of molecular libraries. *Comb. Chem. in Bio*. 243: 159-172.

EPA (2007). Revised N-methyl carbamate cumulative risk assessment (CRA).

Evans, DA and Lawson, KR (1991). Crop protection chemicals-research and development perspectives and opportunities. *Pestic Outlook* 3: 10-15.

Felder CE, Harel M, Silman, I and Sussman, JL (2002). Structure of a complex of the potent and specific inhibitor BW284C51 with Torpedo californica acetylcholinesterase. *Acta Crystallogr. D Biol. Crystallogr.* 58: 1765-1771.

Fournier D, Bride JM, Hoffmann F, Karch F (1992). Acetylcholinesterase: two types of modifications confer resistance to insecticide. *J. Biol Chem.* 267: 14270-14274.

Gallo MA and Lawryk NJ (1991). Organic phosphorus pesticides. *In* Handbook of Pesticide Toxicology. Hayes, W. J., Jr. and Laws, E. R., Jr., Eds. Academic Press, NY. pp 3-5.

Georghiou GP (1994). Principles of insecticide resistance management. Phytoprotection 75: 51-59.

Gless RD, Fisher KJ, and James DR (2001). Combinatorial chemistry in the agrochemical discovery process at Zeneca. *ACS Symp. Ser*. 774: 214-225.

Goosey, MW (1992). Towards the rational design of crop protection agents. *Pestic. Sci*., 34: 313-316.

Grant DF, Dietze EC, Hammock BD (1991). Glutathione S-transferase isozymes in *Aedes aegypti* purification, characterization, and isozyme-specific regulation. *Insect Biochem* 21: 421-33.

Gubler DJ and Clark GG (1995). Dengue/dengue hemorrhagic fever: the emergence of a global health problem. *Emerg. Infect. Dis.* 1: 55-57.

Haller HL, La Forge FB and Sullivan WN (1942). Some compounds related to sesamin: Their structures and their synergistic effects with pyrethrum insecticides. *J. Org. Chem.* (7: 185-188).

Harel M, Kryger G, Rosenberry TL, Mallender WD, Lewis T, Fletcher RJ, Guss JM, Silman I and Sussman JL (2000). Three-dimensional structures of Drosophila melanogaster acetylcholinesterase and of its complexes with two potent inhibitors. *Protein Sci.* 9(6): 1063-1072.

Haviv H, Wong DM, Silman I, and Sussman, JL (2007). Bivalent ligands derived from Huperzine A as acetylcholinesterase inhibitors. *Curr. Top. Med. Chem.* 7: 375-387.

Hayes JD, and Pulford DJ (1995). The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem. Mol. Biol.* 30: 445-600.

Hemingway J, Miyamoto J, Herath PRJ, SJ (1991). A possible novel link between OP and DDT insecticide resistance in Anopheles: supporting evidence from fenitrothion metabolism studies. *Pestic. Biochem. Physiol.* (39: 49-56).

Hemingway J and Georghiu GP (1983). Studies on the acetylcholinesterase of Anopheles albimanus resistant and susceptible to organophosphate and carbamate insecticides. *Pestic. Biochem. Physiol.* 19: 167-171.

Hemingway J and Ranson H (2000). Insecticide resistance in insect vectors of human disease. *Ann. Rev. Entomol.* 45: 369–389.

Hemingway J, Field L, and Vontas J (2002). An overview of insecticide resistance. *Science* 298: 96-97.

Hennesay DJ, Frantanoni J, Hartigan J, Moorfield HH and Weiden MHJ (1961). Toxicity of 2-(2-halogen-4 chlorophenyl)-2-(4 chlorophenyl) 1,1,1-trichloroethane to normal and resistant houseflies. Nature 190 (341).

Hirshhorn N, (1993). Study of the occupational health of Indonesian farmers who spray pesticides. The Indonesian National IPM Program, FAO, Jakarta. 23: 12-16.

Jencks WP (1981). On the attribution and additivity of binding energies. *Proc. Natl. Acad. Sci. USA* 78: 4046-4050.

Kiss Z and Fazekas T (1979). Arrhythmias in organophosphate poisonings. *Acta Cardiol*. 34: 323-330.

Kozaki T, Shono T, Tomita T, Kono Y (2001). Fenitroxon insensitive acetylcholinesterase of the housefly, Musca domestica associated with point mutations. *Insect Biochem. Mol. Biol.* 31: 991-997.

Krogstad DJ (1996). Malaria as a reemerging disease. *Epidem. Rev.* 18: 77-89.

Lands AM, Hoppe JO, Arnold A and Kirchner FK (1958). An investigation of the structure-activity correlations within a series of ambenonium analogs. *J. Pharmacol. Exp. Ther*. 123: 121-127.

LeBlanc FN, Benson BE and Gilg AD (1986). A severe organophosphate poisoning requiring the use of an atropine drip. *J. Toxicol. Clin. Toxicol*. 24: 69-76.

Lengeler C. (1998). Insecticide treated bednets and curtains for malaria control (Cochrane Review). In: The Cochrane Library vol 3. Oxford: Update Software.

Lewis WG, Green LG, Grynszpan F, Radic Z, Carlier PR, Taylor P, Finn MG and Sharpless KB (2002). Click Chemistry In Situ: Acetylcholinesterase as a reaction vessel for the selective assembly of a femtomolar inhibitor from an array of building blocks. *Angewandte Chemie*. 41: 1053-1057.

Lindsay, SW, Emerson P, and Charlwood JD (2002). Reducing malaria by mosquito-proofing homes. *Trends in Parasitology*, 18: 510-514.

Magnus W. (2002). From serendipity to design: Making agrochemicals to order. *Nat. Prod. Reports* 19: 278–291.

Majori G, Sabatinelli G, Villani F and Petrarca V (1986). Studies on insecticide susceptibility of *Anopheles gambiae* s.1. and *Culex quinquefasciatus* in the area of Ouagadougou, Burkina Faso (West Africa). *J. of the American Mosq. Cont. Assoc*. 2: 305-309.

Maitra S, Dombroski SM, Waters LC, Ganguly R (1996). Three chromosome-linked clustered Cyp6 genes show differential constitutive and barbital-induced expression in DDT-resistant and susceptible strains of *Drosophila melanogaster*. *Gene* 180: 165-71.

McCarroll L, Paton MG, Karunaratne SHPP, Jayasuryia HTR, Kalpage KSP and Hemingway J (2000). Insecticides and mosquito-borne disease. *Nature* 407: 961–962.

Menn JJ and Henrick CA (1981). Rational and bio-rational design of insecticides. *Philosop. Trans. of the Royal Soc*., London, Series B 295: 57-71.

Menozzi P, AnShi M, Lougarre1A, HuaTang Z and Fournier D (2004). Mutations of acetylcholinesterase which confer insecticide resistance in *Drosophila melanogaster* populations. *Evol. Biol.* 4: 4-11.

Metcalf RL (1967). Mode of action of insecticide synergists. Ann. Rev. Entomol. 12: 229-256.

Mittal PK (2003). Biolarvicides in vector control: challenges and prospects. *J. Vect. Borne Dis.* 40: 20-32.

Mouches C, Pauplin Y, Agarwal M, Lemieux L, Herzog M and Abadon M (1990). Characterization of amplification core and esterase B1 gene responsible for insecticide resistance in Culex. *Proc. Natl. Acad. Sci. USA.* 87: 2574-2578.

Mutero A, Pralavorio M, Bride JM and Fournier D (1994). Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. *Proc. Natl. Acad. Sci. USA.* 91: 5922-5926.

Mutunga JM, Lomo P, Osir EO, Beier JC, Githure JI and Yan G (2005). Prevalence of kdr gene in *An. gambiae* and *An. funestus* populations from Kenya. Proc. 4th Pan-African Multilateral Initiative of Malaria (MIM) Yaoundé, in *Acta Trop. 95* Suppl: S1-506 pp S362.

N'guessan R, Darriet F, Guillet P, Carnevale P, Traore-Lamizana M, Corbel V, Koffi A.A, Chandre F (2003). Resistance to carbosulfan in *Anopheles gambiae* s.s. from Ivory Coast based on reduced sensitivity of acetylcholinesterase. *Med. Vet. Entomol.* 17:1-7.

Nabeshima T, Mori A, Kozaki T, Iwata Y, Hidoh O, Harada S, Kasai S, Severson DW, Kono Y and Tomita T (2004). An amino acid substitution attributable to insecticide-insensitivity of acetylcholinesterase in a Japanese encephalitis vector mosquito. Culex tritaeniorhynchus, *Biochem. Biophys. Res. Commun.* 313: 794–801.

Newcomb RD, Campbell PM, Ollis DL, Cheah E, Russel RJ, Oakeshott JG (1997). A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on blowfly. *Proc Natl Acad Sci USA*, 94:7464-7468.

NLM (1992). National Library of Medicine hazardous substance databank. *TOXNET*, *Medlars management Section*, Bethesda, MD.

Oakeshott JG, van Papenrecht EA, Boyce TM, Healy MJ, Russell RJ (1993). Evolutionary genetics of Drosophila esterases. *Genetica* 90: 239-68.

O'Brien RD (1967). Insecticides, Action and Metabolism, Academic Press, New York, pp332.

Oppernoorth FJ, Van der Pas LTJ and Houx NWH (1979). GSTs and hydrolytic activity in tetrachlorovinphos-resistant strain of housefly and their influence on resistance. *Pestic. Biochem. Physiol.* 11: 176-188.

Oztoxics report (1986). http://www.oztoxics.org/index.html, Annual report.

Pang Y-P (2006). Novel acetylcholinesterase target site for malaria mosquito control. PLoSONE 1(1): e58. doi:10.1371/journal.pone.0000058.

Patel R, Sanders R, Brown L, Baker S, Tsigelny I and Pezzementi L (2006). A tryptophan in the bottleneck of the catalytic gorge of an invertebrate acetylcholinesterase confers relative resistance to carbamate and organophosphate inhibitors *Cell Biochem. Biophy.* 46: 253-264.

Penilla P, Rodríguez AD, Hemingway J, Torres JI, Arredondo-jiménez JI and Rodríguez MH (1998). Resistance management strategies in malaria vector mosquito control. Baseline data for a large-scale field trial against I in Mexico. *Med. Vet. Ent.* 2(3): 217–233.

Pillmoor JB, Wright K and Lindell SD (1991) Herbicide discovery through rational design: some experiences. In *the proceedings of the Brighton Crop Protection Conference – Weeds*. pp 857.

Prapanthadara L, Koottathep S, Promtet N, Hemingway J, Ketterman AJ (1996). Purification and characterization of a major glutathione S-transferase from the mosquito *Anopheles dirus* (species B). *Insect Biochem. Mol. Biol.* 26: 277-85.

Ranson H and Hemingway J (2004). Insect pharmacology and control: glutathione S-transferases. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.). Elsevier Ltd, Oxford, UK.

Ranson H, Prapanthadara L and Hemingway J (1997). Cloning and characterization of two glutathione S-transferases from a DDT-resistant strain of *Anopheles gambiae*. *Biochem. J.* 324: 97-102.

Ranson H, Jensen B, Wang X, Prapanthadara L, Hemingway J and F.H. Collins (2000). Genetic mapping of two loci affecting DDT resistance in the malaria vector *Anopheles gambiae*. *Insect Mol Biol.* 9: 499–507.

Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, and Corbel V (2010) Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trend. Parasitol.* 27(2): 91-98.

Roberts DR, Laughlin LL, Hsheih P, Legters LJ. (1997). DDT, global strategies and a malaria control crisis in South America. *Emerg. Infect. Dis.* 3: 295-302.

Rydberg EH, Brumshtein B, Greenblatt HM, Wong DM, Shaya D, Williams LD, Carlier PR, Pang YP, Silman I, Sussman JL (2006). Complexes of alkylene-linked tacrine dimers with *Torpedo californica* acetylcholinesterase: Binding of bis(5)-tacrine produces a dramatic rearrangement in the active-site gorge. *J. Med. Chem.* 49(18): 5491-500.

Savini L, Gaeta A, Fattorusso C, Catalanotti B, Campiani G, Chiasserini L, Pellerano C, Novellino E, McKissic D, and Saxena A. (2003) Specific targeting of acetylcholinesterase and butyrylcholinesterase recognition sites. Rational design of novel, selective, and highly potent cholinesterase inhibitors. *J. Med. Chem.* 46: 1-4.

Shen T, Tai K, Henchman RH and McCammon JA (2002). Molecular dynamics of acetylcholinesterase. *Acc. Chem. Res.* 35: 332–340.

Shousha AT (1948). The eradication of *Anopheles gambiae* from Upper Egypt 1942-1945. Bull. World Health Organisation. 1: 309-342.

Silman I and Sussman JL (2005). Acetylcholinesterase: 'Classical' and 'Non-Classical' Functions and Pharmacology. *Curr. Opin. Pharmacol.* 5: 293-302.

Smith (2002). Ensemble Discovery in pesticide design Pesticide Outlook. 13: 21–25.

Soreq H and Seidman S (2001). Acetylcholinesterase-new roles for an old actor. *Nature Rev. in Neurosci.* 4: 294-302.

Stump AD, Atieli FK, Vulule JM and Besansky NJ (2004). Dynamics of the pyrethroid knockdown resistance allele in western Kenyan populations of *Anopheles gambiae* in response to insecticide treated bed-net trials *Am. J. Trp. Med. Hyg.* 70(6): 591-596.

Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L, Silman I (1991). Atomic structure of acetylcholinesterase from Torpedo californica: a prototypic acetylcholine-binding protein. *Science.* 253: 872–879.

Sussman, J. L., Abola, E. E., Lin, D., Jiang, J., Manning, N. O. & Prilusky, J. (1999). The protein data bank. *Genetica.* 106: 149-158.

Taylor P and Radic, Z (1994). The Cholinesterases: From Genes to Proteins. *Ann. Rev. Pharmacol. Toxicol.* 34: 281-320.

ter Kuile FO, Terlouw DJ, Phillips-Howard PA, Hawley WA, Friedman JF, Kariuki SK, Shi YP, Kolczak MS, Lal AA, Vulule JM and Nahlen BL (2003). Reduction of malaria during pregnancy by permethrin-treated bed nets in an area of intense perennial malaria transmission in western Kenya. *Am. J. Trop. Med. Hyg.* 68 (4): 50–60.

Thiermann H, Szinicz L, Eyer F, Worek F, Eyer P and Felgenhauer N (1999). Modern strategies in therapy of organophosphate poisoning. *Toxicol. Lett.* 107: 233-239.

Toda N, Tago K, Marumoto S, Takami K, Ori M, Yamada N, Koyama K, Naruto S, Abe K, Yamazaki R, Hara T, Aoyagi A, Abe Y, Kaneko T, Kogenet H (2003). Design, synthesis and structure-activity relationships of dual inhibitors of acetylcholinesterase and serotonin transporter as potential agents for Alzheimer's disease. *Bioorg. Med. Chem.* 11: 1935-1955.

Tomlin C (Ed.) (2000). The Pesticide Manual. British Crop Protection Council (BCPC), Farnham, UK. 12: 151-160.

Touré YT (1984). Sensitivity of *Anopheles gambiae* s.1. to insecticides in the Selingue dam area. *Parassitologia*. 26: 311-318.

Vaughan A, Hawkes N and Hemingway J (1997). Co-amplification explains linkage disequilibrium of two mosquito esterase genes in insecticide-resistant *Culex quinquefasciatus*. *Biochem. J*. 325: 359-365.

Vontas JG, Hejazi MJ, Hawkes NJ, Cosmidis N, Loukas M and Hemingway (2002). Resistance-associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly, Bactrocera oleae. *Insect Mol. Biol*. 11: 329-336.

Vulule JM, Beach RF, Atieli FK, McAllister JC, Brogdon WG, Roberts JM, Mwangi RW and Hawley WA (1999). Elevated oxidase and esterase levels associated with permethrin tolerance in Anopheles gambiae from Kenyan villages using permethrin-impregnated nets. *Med. Vet. Entomol*. 13: 239-244.

Vulule JM, Beach RF, Atieli FK, Roberts JM, Mount DL and Mwangi RW (1994). Reduced susceptibility of Anopheles gambiae to permethrin associated with the use of permethrin of impregnated bednets and curtains in Kenya. *Med. Vet. Entomol*. 8: 71-75.

W.H.O. (1992). Vector resistance to insecticides: 15th report of the WHO expert committee on vector biology and control. World Health Organ. *Tech. Rep. Ser* 818: 1-62.

W.H.O. (2000). WHO expert committee on malaria. World Health Organ. *Tech. Rep. Ser.* 892: 1–71.

W.H.O. (2002b). Roll Back Malaria. Scaling-up insecticide-treated netting programmes in Africa. A strategic framework for coordinated national action. Geneva, (WHO/CDS/RBM/2002.43).

W.H.O. (2003). The World Health Report 2003: shaping the future. World Health Organization-Geneva.

Walsh SB, Dolden TA, Moores GD, Kristensen M, Lewis T, Devon- shire AL, Williamson MS (2001). Identification and characterization of mutations in housefly (Musca domestica) acetylcholinesterase involved in insecticide resistance. *Biochem J*. 359: 175-181.

Weill M, Fort P, Berthomieu A, Dubois MP, Pasteur N and Raymond M (2002). A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is nonhomologous to the ace gene in Drosophila. *Proc. R. Soc. Lond* B. 269: 2007-2016.

Weill M, Lutfalla G, Mogensen K, Chandre F, Berthomieu A, Berticat C, Pasteur N, Philips A, Fort P and Raymond M (2003). Insecticide resistance in mosquito vectors. *Nature* 423: 136-137.

Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, Marquine CM and Raymond M (2004). The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. *Insect Mol. Biol*. 13:1-7.

Wilce MC and Parker MW (1994). Structure and function of glutathione S-transferases. *Biochim. Biophys. Acta*. 1205: 1-18.

Wilkinson CF (1976). Insecticide biochemistry and physiology. New York: Plenum Press; pp 768-721.

Winkler DA and Holan G (1992). QSAR and rational design of insecticides. 'Rational approaches to structure, activity and ecotoxicology of agrochemicals', Fujita, T. and Draber,W. (Eds). pp 123-145.

Worek F, Koller M, Thiermann H and Szinicz L (2005). Diagnostic aspects of organophosphate poisoning. *Toxicology*. 214(3): 182-9.

Zhou Z-H and Syvanen M. (1997). A complex glutathione transferase gene family in the housefly Musca domestica. *Mol. General Genet.* 256: 187-94.

Chapter 2. Comparative Biochemical Effects of *Bis*(n)-Tacrines on *Blattella germanica* and *Drosophila melanogaster* Acetylcholinesterase

Abstract

I used *bis*(n)-tacrines to probe the acetylcholinesterase (AChE) active site of *Blattella germanica* and *Drosophila melagogaster*, which express AChE-1 and AChE-2 isoforms, respectively. Overall potency of *bis*(n)-tacrines was greater in *D. melanogaster* AChE (*Dm*AChE) than in *B. germanica* AChE (*Bg*AChE). Change in potency with tether length was high in *Dm*AChE and low in *Bg*AChE, associated with 90-fold and 6-fold maximal potency gain, respectively, compared to the tacrine monomer. The two species differed by only about 2-fold in their sensitivity to tacrine monomer, indicating that greater differential potency occurred among dimeric *bis*(n)-tacrines with changing tether lengths. Optimal tether length for *Blattella* was 8 carbons and for *Drosophila* was 10 carbons. Greater potency differences at longer tethers indicate structural differences in AChE active site gorge geometry between the two insect species. Electrophysiological studies on *D. melanogaster* central nervous system show that dimeric tacrines apparently do not cross the blood brain barrier, explaining the observed non-lethality to insects. Although the compounds were not lethal to insects and hence not good insecticide candidates, the information obtained in this study helps inform the design of selective bivalent ligands targeting insect pests and disease vectors. Possible application of the results to bivalent insecticide design is discussed.

2.1 Introduction

Termination of nerve impulses at cholinergic synapses is by a rapid hydrolysis of the neurotransmitter acetylcholine (ACh) by a highly efficient enzyme, acetylcholinesterase (AChE, EC 3.1.1.7) (Sussman *et al.* 1991; Toutant 1989). AChE is the target of carbamate and organophosphate insecticides that form a major group of chemicals used for control of insect crop pests and disease vectors. The enzyme is found in both vertebrates and invertebrates, and different isoforms exist. Vertebrate AChE is encoded by several genes induced by alternative splicing, but only *ace*-1 is physiologically relevant in vertebrate synaptic transmission (Silver, 1974, Massoulie and Bon, 1982). Invertebrates, on the other hand, have both *ace*-1 and *ace*-2, with either of them being important in synaptic transmission for a given species (Weill *et al.* 2002, Li and Han, 2002, Huchard *et al.* 2006, Mori et al 2007). Consequently, the organophosphate (OP) and carbamate (CB) insecticides target either AChE-1 or AChE-2 in insects, depending upon which isoform is involved in synaptic transmission.

Established carbamate insecticides, such as propoxur (Baygon®) have low selectivity for insects (ca. 10-20 fold) and therefore pose the threat of toxicity to humans and the environment (Carson and Wilson 1962; Goldstein *et al.* 1999; Walker 1983). The adoption of the Food Quality Protection Act by the U.S. Environmental Protection Agency (EPA) has led to de-registration or re-registration of insecticides. Re-registration involves revising labels to exclude them from indoor use purposes, as has been done with bendiocarb (EPA fact sheet 0409; 1999). With the limited availability of safe carbamates and organophosphate insecticides for use in insect disease vector control, there is an urgent need for new insecticides to avert emerging threats of disease from pest control failures.

Despite the shortcomings associated with current anti-AChEs, fine-scale molecular analysis of AChE structure provide insights on possible ways to design inhibitors that are safer and unlikely to select for resistance. Critical to this approach is the discovery of the bivalent nature of AChE mediated by two active sites in the enzyme. The AChE active site is a 20 Å deep narrow gorge with a peripheral anionic site (PAS) at the mouth of the gorge and the catalytic active site (CAS) at the bottom of the gorge (Sussman *et al.* 1991). Both PAS and CAS have been shown to act in concert and allosterically during the hydrolysis of the substrate, acetylcholine (ACh) (Bourne *et al.* 1999; Bui *et al.* 2003; Haviv *et al.* 2005; Mallender *et al.* 2000). The hydrolysis process starts with ACh binding to the PAS, inducing a conformational change that orients the CAS into a position ideal for the catalytic process. The substrate then proceeds down the gorge and binds the catalytic triad at the CAS, forming a tetrahedral complex and resulting in hydrolysis (Zhang *et al.* 2002). All the current anticholinesterases target a conserved Ser_{200} (*Torpedo californica* numbering), within the catalytic triad of the CAS (Cohen and Oosterbaan, 1963; Wilson *et al.* 1960). With the advent of new technologies in protein chemistry and computational biology, it is possible to perform fine-scale modeling of AChE, to further understand functional and structural differences that may be utilized in design of new insecticides. Our findings provide insights into differences in AChE-1 and 2, with focus on optimal molecular spacing of bivalent ligands along the AChE gorge. The data will aid in design of bivalent insecticides with improved selectivity, less likely to induce insecticide resistance, and be safer to humans and other non-target species.

2.2 Materials and methods

2.2.1 Chemicals and insects

Tacrine monomer (tetrahydroaminoacridine, or THA), and *bis*(n)-tacrines (Fig. 2.1) were synthesized to 99.5% purity using established methods (Carlier *et al.* 1999; Wang *et al.* 1999). Ellman reagents acetylthiocholine iodide (AChI), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) and buffer components were purchased from Sigma (Sigma Chemical Co. St. Louis, MO, USA). *D. melanogaster* and *B. germanica* adult females were obtained from insecticide-susceptible Oregon-R and CSMA strains, respectively, both maintained at the Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061, USA.

Figure 2. 1. Chemical structure of the *bis*(n)-tacrine used in the assay in this study. The 'n' represents number of carbons that form the tether linkage.

2.2.2   Enzyme activity measurements

Adult female insects were stored at -80 ^{0}C before use. I used insect head homogenate for *B. germanica* and whole insect homogenate of *D. melanogaster*, as enzyme sources for inhibition assays. Insect tissues were homogenized in 1 mL of 0.1 M phosphate buffer containing 0.1 M phosphate and Triton X-100, using a glass tissue homogenizer.   To achieve maximal AChE activity, the buffer pH and Triton X-100 concentration were optimized at pH 7.8 and 0.3% v/v for *Dm*AChE and at pH 8.0 and 1.5% for *Bg*AChE. Homogenates were centrifuged for 5 min at 10,000 × *g* and 4 °C in a Sorvall Fresco refrigerated centrifuge (Thermo Electronics Co., Germany), and the supernatant used as source of AChE for a 96-well microplate assay. AChE activity was measured by the method of Ellman *et al.* (1961), where the hydrolysis of acetylthiocholine iodide was determined colorimetrically by absorbance of DTNB-thiocholine complex at 405 nm. The ACTh and DTNB concentrations were optimized at 0.4 mM and 0.3 mM, respectively.   Potency of *bis*(n)-tacrines on AChEs was determined by incubating the enzymes under different concentrations of the inhibitor (10^{-5} M to 10^{-10} M) for 10 min. Compounds were dissolved in DMSO, and control enzyme activity was determined in 0.1% DMSO. After addition of substrate and indicator, AChE activity was measured for 10 min at 25 °C in a DYNEX *Triad* microplate reader (DYNEX Technologies, Chantilly, VA, USA). Data were corrected for background absorbance, and residual enzyme activities were converted to percent of control and analyzed by nonlinear regression to a four parameter logistic equation to determine IC$_{50}$ values and 95% confidence limits (CL) using Prism™ (GraphPad Software, La Jolla, CA, USA).

2.2.3 Microinjection studies and insect bioassays

Toxicity experiments were performed with the most potent *bis*(n)-tacrine being used for each case. For *D. melanogaster*, twenty five insects per concentration were treated with varying concentrations of *bis*(10)-tacrine in sucrose solution using cotton wicks. Similarly, I screened *B. germanica* adult females by applying 0.5 µl *bis*(8)-tacrine at varying concentrations onto the insect abdomen. Microinjections were performed by injecting 500 nL of 1 mM *bis*(n)-tacrines into the abdomen of *B. germanica*. In all bioassays, mortality was recorded 24 hr post treatment.

2.2.4 Electrophysiological studies of Drosophila *CNS*

Electrophysiology experiments were conducted by Dr. Raj Boina, who was a fellow student in Dr. Bloomquist's laboratory, and I used spike frequency data to quantify the effects of desheathed *Drosophila* CNS. Electrophysiological recordings were performed on third instar larvae of *D. melanogaster* as described previously (Bloomquist *et al.* 1992) with slight modifications. Peripheral nerve activity was recorded, *in vitro*, from transected or intact ventral ganglia with a micropipette suction electrode. Signals were displayed and analyzed on a microcomputer system designed to emulate a 2-channel chart recorder (PowerLab™, SDInstruments, Colorado Springs, CO, USA). Average spike rates were calculated, on line, before and after drug treatment, and compared by ANOVA and Tukey's post test (Instat™, GraphPad Software, La Jolla, CA, USA).

2.2.5   AChE sequence alignments

Alignments for the catalytic subunits of *Tc*AChE, *Bg*AChE, and *Dm*AChE were performed using CLUSTAL W (1.83) multiple sequence alignment (Chenna *et al.* 2003; Larkin *et al.* 2007) on the full-length precursor sequences (SwissProt codes ACES_TORCA, Q2PZG3_BLAGE, and ACES_DROME respectively). Alignments were then cross-checked against the published, crystallized mature forms of *Tc*AChE (PDB ID 2ACE) (Raves *et al.* 1997; Millard *et al.* 1999) and *Dm*AChE (PDB ID 1dx4) (Harel *et al.* 2000). The alignment of the flexible peripheral site loop, around the deletion region for *Bg*AChE and *Dm*AChE were manually adjusted, by aligning G287(*Bg*) with S286(*Tc*), and S335/G336(*Dm*) with D285/S286(*Tc*). By convention, the main catalytic subunit of *Bg*AChE (Q1-N544) was numbered based on the mature form of *Tc*AChE, beginning at residue Q109 from the full-length sequence, Q2PZG3_BLAGE (*Bg* ace-1). Unless otherwise stated, sequence identities and similarities to *Bg*AChE were calculated using the BLAST 2 sequence program (bl2sq) from NCBI, with default settings (Tatusova and Madden, 1999), and based on alignments of *h*AChE, *m*AChE, and *Dm*AChE against Q1-N544 of *Bg*AChE, which corresponds to the mature catalytic subunit in *Tc*AChE.

2.2.6   Homology modeling

Homology modeling was done by Dr. Dawn Wong, a postdoctoral research associate in the Department of Chemistry, Virginia Polytechnic Institute and State University. Three-dimensional structures of *Bg*AChE (*Bg* ace-1) were created by automated homology modeling using the program MODELLER (6v2) (Lambert *et al.* 2002), with default settings for all calculations. The structural templates used were *Tc*AChE (PDB ID 1W4L, 2.16 Å) (Greenblatt

et al. 2004), *m*AChE (PDB ID 1N5R, 2.25 Å; chain A) (Bourne *et al.* 2003), and E202Q mutant

*h*AChE (PDB ID 1F8U, 2.90 Å) (Kryger *et al.* 2000). The *h*AChE template was chosen by the

best-fit option of Modeller (6v2), which shares 35.9% sequence identity with *Bg*AChE (using the

ALIGN program therein). By contrast, the *Tc*AChE and *m*AChE templates used for homology

modeling were chosen owing to their high resolution structural data, which share 35.3% and

34.6% sequence identity with *Bg*AChE, respectively (using the ALIGN program therein). After

accounting for gaps and insertions, all templates possess about 57%-88% sequence identity with

each other, and 43-44% sequence identity with *Bg*AChE. No subsequent refinements were

made.

2.3 Results

2.3.1   *Responses to* Bis*(n)-tacrines differ in* Drosophila *and cockroach AChE*

We used percent residual AChE activity to plot sigmoidal dose-response curves for each

inhibitor in both species (Fig. 2.2). THA and *bi*s(n)-tacrines are complete inhibitors of AChE,

with potencies that are dependent upon tether length, with different optimal tether lengths for

Drosophila and *Blattella*. Compared to the monomeric THA, optimum tether length for

Blattella was 8 carbons and for *Drosophila* was 10 carbons.
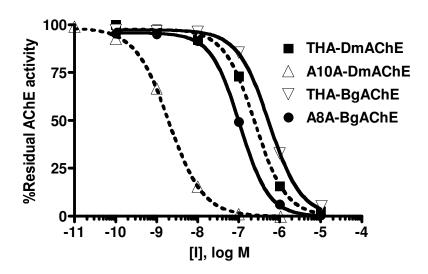
Figure 2. 2. Sigmoidal plots showing the concentration-dependent inhibition of *Dm/Bg*AChEs by THA and their respective most potent *bis* (n)-tacrine.

The values of IC_{50} and their 95% confidence limits for the complete range of dimeric tacrines tested are shown in Table 2.1, where final solvent concentration was maintained at 0.1% DMSO in all assays, and the Hill slope and R^2 values for the curve fit were greater than 1 and 0.99; respectively. IC_{50} values are also presented graphically in Fig. 2.3 to better enable visualization of the structure-dependent pattern of tether length dependence. Statistical significance was assessed based on non-overlapping 95% confidence intervals (P< 0.05). Tacrine and its dimeric forms of 2-12 carbon linkers are micromolar to nanomolar inhibitors of *Bg* and *Dm* AChEs. In the cockroach, *bis*(12)-tacrine had the lowest potency, followed by *bis*(2), (3) and (4), all of which are less potent than the monomer, tacrine. Overall, the tether-length dependence observed for *Blatella* across all *bis*(n)-tacrines had a "U" shaped relationship, with a bump at *bis*(2)-tacrine, associated with a ca. 4-fold loss in potency (Fig. 2.3). Despite the loss of potency at C_2, the potency of *bis*(3)-tacrine is comparable to that of the monomer, after

which potency increases up to the maximum at *bis*(8)-tacrine. In contrast, the plot for *Drosophila* shows declining IC_{50}s with increasing tether length, with bumps at *bis*(2)- and *bis*(4)-tacrine, along with a general plateau occurring from *bis*(5)-tacrine to *bis*(12)-tacrine (Fig. 2.3). Within the plateau region, the differences in *Dm*AChE IC_{50}s are not statistically significant (P > 0.05).

Based on the IC_{50} values, I computed the tether length dependence index (TLDI, Table 2.1) of both AChEs by calculating the IC_{50} ratio of *bis*(n)-tacrine (numerator) versus that of tacrine monomer (denominator). The most potent tacrine dimer to *Bg*AChE and *Dm*AChE were *bis*(8)- and *bis*(10)-tacrine, respectively, accounting for a 39-fold and 90-fold TLDI compared to the monomer (Table 2.1). The C_2 and C_4 bumps in *Dm*AChE present 1.7-fold and 2-fold decreases in potency, with the highest potency loss of 152-fold occurring between *bis*(2)- and *bis*(10)-tacrine, compared to only 27-fold in *Bg*AChE. *Dm*AChE had only *bis*(2)-tacrine with a TLDI value <1, meaning that potency was lower than with monomer. In contrast, there are four such values in *Bg*AChE namely; *bis*(2)-, (3)-, (4)- and (12)-tacrine.

Following the observed overall enhanced potency to *Dm*AChE compared to *Bg*AChE, I computed the potency ratio (PR: *Bg* IC_{50}/*Dm* IC_{50}) for each tacrine dimer (Table 2.1). Enhanced *Dm*AChE potency compared to *Bg*AChE increased with tether length, and the difference is maximal at *bis*(12)-tacrine (PR= 297). At *bis*(10)-tacrine, potency to *Dm*AChE is 137-fold higher than in *Bg*AChE; the second highest difference observed in this study (Table 2.1).

Table 2. 1. IC_{50} values and tether length dependence indices of tacrine dimers.

Compound	IC_{50}, BgAChE*	IC_{50}, DmAChE*	TLDI-BG[#]	TLDI-DM[#]	PR[#]
Tacrine (THA)	556 (524-589)[a,1]	242 (221-265)[a,2]	1	1	2.3
Bis(2)-tacrine	1969 (1885-2056)[b,1]	411 (341-500)[b,2]	0.3	0.6	4.5
Bis(3)-tacrine	799 (737-866)[c,1]	25 (22-28)[c,2]	0.7	9.7	32
Bis(4)-tacrine	781 (730-836)[c,1]	56 (52-62)[d,2]	0.7	4.3	14
Bis(5)-tacrine	472 (444-503)[d,1]	6.2 (5.6-7.0)[e,f,2]	1.2	39	76
Bis(6)-tacrine	243 (232-254)[e,1]	5.5 (5.1-5.8)[e,2]	2.3	44	44
Bis(7)-tacrine	115 (84-157)[f,1]	7.5 (6.5-8.8)[f,i,2]	4.8	32	15
Bis(8)-tacrine	106 (103-109)[f,1]	6.2 (5.8-6.8)[e,f,g,2]	5.8	39	17
Bis(9)-tacrine	235 (191-290)[e,1]	5.4 (4.7-6.3)[e,g,2]	5	45	44
Bis(10)-tacrine	371 (343-402)[g,1]	2.7 (2.4-3.6)[h,2]	1.5	90	137
Bis(12)-tacrine	2880 (2507-3303)[h,1]	9.7 (8.6-11.3)[i,2]	0.2	25	297

*IC_{50}, nM with 95% CI in parentheses. Columns and rows bearing the same letter or number; respectively, are not statistically significant based on non-overlapping 95% CI. Final solvent concentration was maintained at 0.1% v/v DMSO in all assays; the Hill slope and R^2 values were greater than 1 and 0.99; respectively. [#]TLDI-BG and TLDI-DM = (IC_{50} of monomer)/(IC_{50} of *bis*(n)-tacrine) for *Bg*AChE and *Dm*AChE, respectively. Potency ratio (PR) = (IC_{50} with *Bg*AChE)/(IC_{50} with *Dm*AChE) indicating the selectivity in potency of *D. melanogaster* by dimeric tacrines compared to *B. germanica*.
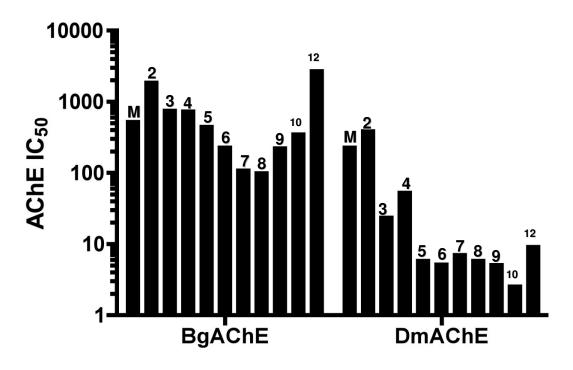
Figure 2. 3. Plots of AChE IC_{50}s (Y-axis) across *bis* (n)-tacrines for *B. germanica* and *D. melanogaster* AChE (X-axis). M = tacrine monomer, and numbers indicate tether length in carbon atoms (e.g., 2 = *bis* (2)-tacrine, etc).

2.3.2. *Dimeric tacrines do not cross the insect blood brain barrier,* in vivo

Despite being highly potent inhibitors *in vitro*, neither mortality nor signs of intoxication were observed following ingestion (*Drosophila*) or injection (*Blattella*) bioassays. It was hypothesized that lack of sufficient penetration into the central nervous system (CNS) was at least partly responsible for the lack of toxic effect. Accordingly, peripheral nerve firing rates after *bis*(4)-tacrine in both intact and desheatherd CNS were determined (Fig. 2.4). The transected CNS had a disrupted barrier and *bis*(4)-tacrine had an immediate effect (Fig. 2.4A), while the intact preparations showed no effect of *bis*(4)-tacrine for 15 min of incubation (Fig. 2.4B). Spike frequency values were plotted and analyzed to generate Fig. 2.4C. The only significant *bis*(4)tacrine effect was in the desheathed preparations (Fig. 2.4C).
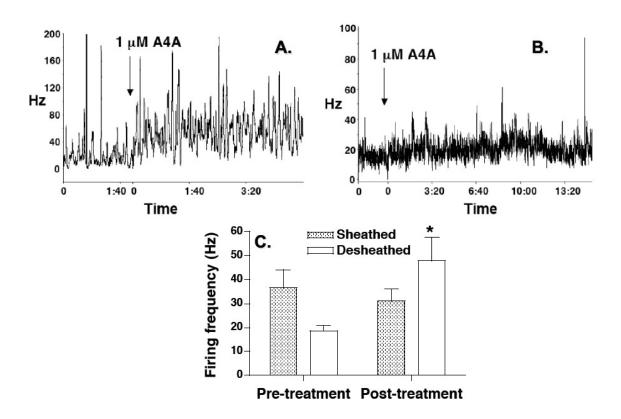
Figure 2. 4. Electrophysiological recordings of transected (A) and intact (B) *Drosophila* CNS treated with *bis*(4)-tacrine, denoted A4A, generated by Dr. Raj Boina, a former student of Dr. Bloomquist. Note the different scale in the X-axis between the 2 graphs. I used data in A and B to perform statistical analysis of spike rates (C) using ANOVA. Asterisk indicates a significant increase in firing rate after *bis*(4)-tacrine treatment to desheathed preparations.

2.3.3. Homology models of D. melanogaster and B. germanica AChEs

Alignments were done for six catalytic active site signatures, namely the catalytic triad, oxyanion hole, choline binding site, acyl pocket, the peripheral site and the peripheral site loop (Fig. 2.5). For consistency purposes, the amino acid numbering is based on *Tc*AChE unless stated otherwise. At the acyl pocket, differences observed from the *Tc*AChE were a F288C in *Bg*AChE and F288L in *Dm*AChE. At the peripheral site, differences observed from the *Tc*AChE were Y70I and G334Y in *Bg*AChE, and Y70E and G334D in *Dm*AChE. Notably, the Y334 and D334 in *Bg* and *Dm*ACHE respectively, involve a change of an aromatic residue to an acidic

residue. Ionic interactions conferred by D334 may contribute to major conformation changes or

rigidity in *Bg*AChE compared to weak pi-pi interactions of the Y334 in *Dm*AChE, which may

allow for more flexibility of the enzyme. This flexibility at the PAS may explain the low change

in *Dm*AChE potencies at longer tethers. The peripheral loop was comprised of a similar 9-12

amino acid residues in the 3 sequences except a C289 in *B. germanica* (*Bg*AChE numbering).

```
          catalytic triad              oxyanion hole          choline-binding site

              200        327  440       116     201             84      330
Tc        TIFGESAGGAS     E    H       YGGGF    A              W       FF
              202        328  442       119     203             87      331
Bg ace-1  TLFGESAGAVS     E    H       FGGGF    A              W       YF
              238        367  480       148     239             83      370
Dm ace-2  TLFGESAGSSS     E    H       YGGGF    A              W       YF


          acyl pocket                 peripheral site          peripheral site loop^b ^a

          233   288  290       70   72  121  279  334       279              291
Tc        W     F    F         Y    D   Y    W    YG        WNVLPFDSIFRFS
          235   289  291       73   75  124  283  335       283              292
Bg ace-1  W     C    F         I    D   Y    W    YY        WGTL---GICEFP
          271   328  330       69   71  153  321  374       321              331
Dm ace-2  W     L    F         E    Y   M    W    YD        WNSY--SGILSFP
```

Figure 2. 5. Alignment of key active-site residues of *T. californica* (*Tc*), *B. germanica* (*Bg ace-*1), and *D. melanogaster* (*ace*-2) AChEs. Residues marked in bold are essential for catalysis, as well as other residues in the acyl pocket and peripheral site that differ in *Bg ace*-1 (see text for explanation). [a]The peripheral site loop region has previously been referred to as the "acyl pocket loop" (Radic and Taylor, 2006) and in *Tc*AChE as the "W279-S291 loop", where its flexibility has been noted (Morel *et al.* 1999; Greenblatt *et al.* 2004; Haviv *et al.* 2005 and Rydberg *et al.* 2006).

From the multiple sequence alignment, I constructed a phylogram (Fig. 2.6) to estimate

the genetic relatedness of the two AChEs used in this study, and compared to *TcAChE*, the

template used for molecular modeling. I also included sequence data for human, bovine and rat

AChE, whose tacrine dimer data is presented in fig. 2.7. As expected, the AChE-1 sequences of

Torpedo (0.20) and *Blattella* (0.17) are more closely related than *Drosophila's* AChE-2 (0.34).

Drosophila AChE clusters away from the other 2 insect AChEs, this agrees with *in vitro* dimeric tacrine data where mosquito and cockroach AChE gave similar pattern of inhibition (Fig. 2.7). *Torpedo* AChE, whose template was used for homology modeling has its genetic distance between that of *Blattella* and *Drosophila*, indicating that it was an ideal template for the molecular modeling. As expected, human, bovine and rat sequences clustered together.


```
                                              ┌──── Anopheles: 0.14089
                                         ┌────┤     Blattela: 0.16988
                                    ┌────┤     └──── Drosophila: 0.34458
                                    │    └──── Torpedo: 0.20179
                            ┌───────┤
                            │  ┌─ Homo: 0.03793
                            └──┤── Bos: 0.03966
                               └── Rattus: 0.05170
```
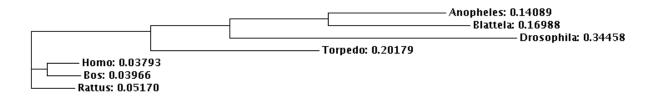
Figure 2. 6. Phylogram showing the genetic relatedness (numbers) of *Blattella* and *Drosophila* AChE sequences, in relation to other insect and vertebrate acetylcholinesterases. Numbers of close values indicate close genetic relatedness of the AChE protein sequences, based on a linear sequence alignment, using Clustal-W.

2.4 Discussion

This study revealed marked differences in the inhibitory trend of tacrine dimers to *Drosophila* and *Blattella* acetylcholinesterases. *Blattella* showed a symmetrical loss and gain in potency with tether length, except for a bump at *bis* (2)-tacrine, associated with a ca. 4-fold loss in potency compared to the monomer. I refer to the bump a C_2-steric bump, which results from crowding of 2 closely linked tacrine units leading to a steric clash of the residues at the CAS and the two aromatic rings of the *bis*(2)-tacrine. Given it had the highest potency in *Blattella,* it is therefore apparent that *bis*(8)-tacrine had maximally favorable ligand-receptor interactions. The initial gain in potency at monomer and short tethers and a loss in potency at longer tethers indicate that our series of ligands spanned the entire *Bg*AChE gorge, interacting with the CAS and PAS residues. The dramatic loss of potency at *bis*(12)-tacrine suggests that the tether linker

was too long and consequently spanned beyond the favorable range of the PAS, causing a possible steric clash at the PAS.

The C_2 bump seems to be conserved in both AChEs, and is joined by a C_4 bump in *Dm*AChE. Longer tethers in *Dm*AChE present a different scenario not observed with *Bg*AChE. Notably, *bis*(12)-tacrine is the least potent in *Bg*AChE, compared to *bis*(2)-tacrine in *Dm*AChE. Moreover, *bis*(12)-tacrine is ca. 4-fold less potent compared to most potent compound for *Dm*AChE, whereas, it is 27-fold less potent in *Bg*AChE. Additionally, the most potent ligand is 2 carbons longer in *Dm*AChE than in *Bg*AChE, and there is no drastic loss in potency at longer tethers in *Dm*AChE, as observed with *Bg*AChE. These observations suggest possible key structural moieties that cause differential ligand-enzyme interactions in the two species. It is unclear if *bis*(12)-tacrine spanned the entire length of the *Dm*AChE gorge (CAS to PAS), since no major loss in potency was observed with this species. Screening *Dm*AChE with dimeric tacrines of longer tethers may resolve this uncertainty.

I computed TLDI values to compare potency of a tacrine dimer relative to the monomer for each of the enzymes. In general, these values are higher in *Dm*AChE than in *Bg*AChE (Table 2.1). This means that change of tether lengths led to higher potency change in *Dm*AChE than *Bg*AChE, synonymous with differential interactions of the ligands between the 2 enzymes. It is possible that the gorge depth, width and flexibility in both species are different. The conserved nature of AChEs in both linear protein sequence and function may not translate to similarity in gorge geometry. My findings call for AChE gorge geometrical considerations in the design of bivalent insecticides for control of insect pests and disease vectors.

I used the PR to decipher the extent to which dimeric tacrines were more potent to *Dm*AChE, compared to *Bg*AChE. High PR values were observed at tethers longer than 5C, with

the highest value at *bis*(12)-tacrine, and correlate positively with increasing tether length (R^2 = 0.5, data not shown). Tacrine monomer had the lowest PR value, which increased with tether length. These findings suggest that tacrine monomer inhibits the two enzymes in a fairly similar way (ca. 2-fold difference), and interactions differ greatly with the dimeric tacrines. The number of steric bumps may relate to the flexibility of the AChE gorge, therefore allowing for maximal orientation of the ligand even at single carbon changes in length. Previous reports have shown that the gorge in AChE-2 is wider than in AChE-1 (Huchard *et al.* 2006), and therefore likely to be more flexible. Our study suggests the need to further evaluate the heterogeneity of the active site gorge across species, and its implications to the molecular design of highly potent, selective and safe bivalent insecticides across pest species.

I compared potency values obtained in our study with other insect and vertebrate data obtained from published literature to make an overall comparison, as shown in Figure 2.7. I observed that the inhibition pattern of mosquito and cockroach AChE is similar. *D. melanogaster* expresses *ace*-2 only (Fournier *et al*. 1993; Mutero *et al*. 1994), whereas *B. germanica*, *An. gambiae,* and vertebrates expresses both *ace*-1 and *ace*-2, with only *ace*-1 being toxicologically relevant (Silver, 1974; Massoulie and Bon, 1982; Li *et al*. 1991). The potency profile of *Drosophila* is similar to that of vertebrates (Fig. 2.7), despite being genetically closely related to the insect species (Fig 2.6). This indicates that selectivity of AChE inhibitors to *Drosophila* may also lead to toxicity in vertebrate systems, and therefore *Dm*AChE is not an ideal model for assessing selectivity of carbamate insecticides to insect pests and disease vectors.

46
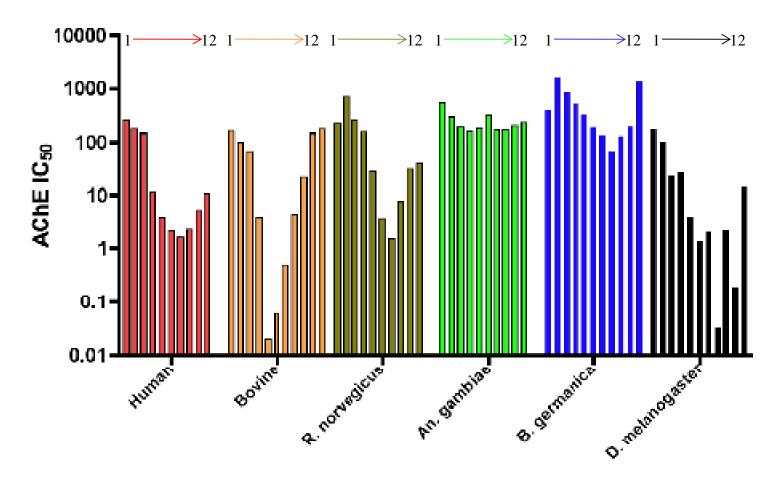
Figure 2. 7. Responses of vertebrate and insect AChEs to inhibition by dimeric tacrines. Human and *An. gambiae* AChE data was obtained from a study by Anderson *et al.* (2009), whereas bovine and rat data were adapted from Munoz *et al.* 2005 and Wang *et al.* 1999; respectively.

In this study, conserved enzyme function is depicted by the closeness of the most potent compounds in each AChE from each species. Previous studies have reported optimal tethers to as *bis*(7)-tacrine in human, *Anopheles gambiae* and rat AChEs; bis(8)-tacrine in *Aedes aegypti*, *Aedes Albopictus* and *Blattela germanica*, and *bis*(6)-tacrine in chicken AChEs (Anderson *et al.* 2009; Camps *et al.* 2005; Mutunga *et al.* 2010; and Wang *et al.* 1999). With *Drosophila* data included, the overall maximal potency range is *bis*(6)-tacrine to *bis*(10)-tacrine, suggesting that the tether length requirement and therefore distance between the CAS and PAS is conserved across species.

Microinjection of these compounds into *B. germanica* adult females was not lethal. Our results agree with the previously reported non-lethality of these compounds to mosquitoes (Anderson *et al.* 2009). Non-lethality of nanomolar AChE inhibitors suggests an underlying barrier of penetration of the chemical to its target, possibly at the blood-brain interface. The possibility of insect cuticle being the only barrier was unlikely, since no mortality was observed in treated wick sugar-feeding assay in *D. melanogaster*. We observed greater firing frequency in the transected (desheathed) CNS compared to the intact CNS (Fig 2.4). This suggests that the blood-brain barrier (BBB) prevented the penetration of dimeric tacrines in to the CNS hence the observed non-toxicity. The presence of a basic charged N-atom in *bis* tacrines (pKa range 8-10; Summers *et al.* 1989; Freeman and Dawson, 1991; Wilson *et al.* 2009) increases polarity and decreases hydrophobicity consequently making them unable to penetrate the BBB. Similar observations were observed with carbamates physostigmine and neostingimine, due to the presence of a charged N atom of quaternary ammonium group (Kolbezen *et al.* 1954).

From the sequence alignments (Fig. 2.5) some key catalytically important residues in the peripheral acyl site, choline binding site, the oxyanion hole and the catalytic triad were observed.

Such key residues maybe suitable targets for designing an insecticide meant to interact preferentially with either of the AChEs, with the aim to scale up selectivity to target pest species. These residues may not act individually in the pharmacological process with the ligand, but in concert with other residues lining the AChE gorge. Previous studies report that dramatic rearrangement of the AChE gorge occurs upon binding of dimeric tacrines (Colletier *et al.* 2006; Rydberg *et al.* 2006), and I conclude that a similar mechanism operate in both *Bg*AChE and *Dm*AChE. The differential interaction of *Dm* and *Bg* AChEs suggest that detailed considerations into the structural enzyme-ligand associations need to be made in the design of bivalent insecticides.

Bivalent insecticides will potentially negate the possibility of resistance development associated with single nucleotide polymorphisms at insecticide target sites. By inhibiting the enzyme at both the PAS and the CAS, multiple mutations will be required to occur in order to confer resistance to a bivalent insecticide. Such multiple mutations would be rare, and if present should confer a great fitness cost to the insect. Other than the G119S mutation, some insects have different mutations associated with OP and CB resistance, such as S431F (S331 in *Torpedo*) in the *ace*-2 of *M. persicae*. The *A. gossypii ace*-1 has A302S (A201 in *T. californica*) found in association with S431F, the two mutations correlate with a much broader resistance to OP and CB insecticides (Li and Han, 2004; Nabeshima *et al.* 2003). The G119S mutation, which confers resistance to carbamates and organophosphates, causes a 30% loss in enzyme activity in *Culex pipiens* (Lenormand *et al.* 1999). The 30% decrease in activity is associated with a substantial fitness cost in this mosquito, about 11% per generation during the breeding season, and 50-60% for survival in the over-wintering season (Lenormand and Raymond, 2000). The mutation also occurs in other mosquito species (e.g.) *An. gambiae* and *An. albimanus,* which

may indicate that AChE function cannot be modified through other alternative mutations without compromising its biological function. A bivalent inhibitor of AChE will interact simultaneously with both the CAS and PAS residues, thereby having more than one amino acid as their target. For resistance to develop for such an insecticide, mutations have to occur at several places within the enzyme active site, a phenomenon that may be physiologically costly for the survival of the insect.

References

Anderson T, Paulson S, Wong D, Carlier P, Bloomquist R (2009) Pharmacological mapping of the acetylcholinesterase catalytic gorge in mosquitoes with bis(n)-tacrines In. Advances in human vector control, ACS Symposium Series, Vol. 1014; Chapter 10, pp 143-151.

Bloomquist JR, Roush RT, and ffrench-Constant RH (1992) Reduced neuronal sensitivity to dieldrin and picrotoxinin in a cyclodiene-resistant strain of *Drosophila melanogaster* (Meigen). Arch. Insect Biochem. Physiol. 19: 17-25.

Bourne Y, Grassi J, Bougis PE, Marchot, P (1999) Conformational flexibility of the acetylcholinesterase tetramer suggested by x-ray chrystallography. J Biol Chem 274: 30370-30376 .

Bourne Y, Taylor P, Radic Z, Marchot P (2003) Structural insights into ligand interactions at the acetylcholinesterase peripheral anionic site. EMBO J. 22: 1-12.

Bui J, Henchman R, McCammon, J (2003) The dynamics of ligand barrier crossing inside the acetylcholinesterase gorge. Biophys. J. 84: 2267-2272.

Camps P, Formosa X, Muñoz-Torrero D, Petrignet J, Badia A, Clos V (2005) Synthesis and pharmacological evaluation of Huprine–Tacrine heterodimers: Subnanomolar dual binding site acetylcholinesterase inhibitors. ACS Med. Chem. Lett. 48(6): 1701-1704.

Carlier P, Han Y, Chow E, Li C, Wang H, Lieu T, Wong H, Pang YP (1999) Evaluation of short-tether bis-THA AChE inhibitors. A further test of the dual binding site hypothesis. Bioorg. Med. Chem. 7: 351-357.

Carson R, Wilson O (1962) Silent Spring. Houghton Mifflin, Boston MA.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson T, Higgins D, Thompson J (2003) Multiple sequence alignment with the Clustal series of programs. Nucl. Acids Res. 31: 3497-3500.

Cohen JA and Oosterbaan RA (1963). The active site of acetylcholinesterase and related esterases and its reactivity toward substrates and inhibitors. *Handbuch der Experimentellen Pharmakologie* 15: 300-373.

Colletier J, Sanson B, Nachon F, Gabellieri E, Fattorusso C, Campiani G, Weik M (2006) Conformational flexibility in the peripheral site of *Torpedo californica* acetylcholinesterase revealed by the complex structure with a bifunctional inhibitor. J. Am. Chem. Soc. 128: 4526-4527.

Dong S, Andrews F, Li F, Moores G, Han Z, Williamson M (2005) Acetylcholinesterase genes and insecticide resistance in aphids. Chem. Biolo. Interac. 14: 373-374.

Ellman G, Courtney K, Andres V, Feather-Stone R (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7: 88-95.

EPA (1999) Bendiocarb re-registration. EPA fact sheet number 738-F-99-010.

Fournier D and Mutero A (1994) Modification of acetylcholinesterase as a mechanism of resistance to insecticides. Comp Biochem Physiol 108: 19-31.

Freeman SE and Dawson RM (1991) Tacrine: a pharmacological review, Prog Neurobiol 36: 257-277.

Goldstein M, Lacher T, Woodbridge B, Bechard M, Canavelli S, Zaccagnini M, Cobb G, Scollon E, Tribolet R, Hooper M (1999) Monocrotophos-induced mass mortality of Swainson's hawks in Argentina, 1995-96. Ecotox 8: 201-214.

Greenblatt H, Guillou C, Guénard D, Argaman A, Botti S, Badet B, Thal C, Silman I, Sussman J (2004) The complex of a bivalent derivative of Galanthamine with *Torpedo* Acetylcholinesterase displays drastic deformation of the active-site gorge: implications for structure-based drug design. J. Americ. Chem. Soc. 126: 15405-15411.

Kolbezen MJ, Metcalf RL, and Fukuto TR (1954) Insecticide structure and activity, insecticidal activity of carbamate cholinesterase inhibitors. J. Agric. Food Chem. 2(17): 864-870.

Harel M, Kryger G, Rosenberry T, Mallender W, Lewis T, Fletcher R, Guss J, Silman I, Sussman J (2000) Three-dimensional structures of *Drosophila melanogaster* acetylcholinesterase and its complexes with two potent inhibitors. Protein Science 9: 1063-1072.

Haviv H, Wong D, Greenblatt H, Carlier P, Pang YP, Silman I, Sussman J (2005) Crystal packing mediates enantioselective ligand recognition at the peripheral site of acetylcholinesterase. J Am Chem Soc 127: 11029-11036.

Huchard E, Martinez M, Alout H, Douzery J, Lutfalla G, Berthomieu A, Berticat C, Raymond M, Weill M (2006) Acetylcholinesterase genes within the diptera: takeover and loss in true flies. Proc R Soc B 273: 2595-2604.

Kryger G, Harel M, Giles K, Toker L, Velan B, Lazar A, Kronman C, Barak D, Ariel N, Shafferman A, Silman I, Sussman J (2000) Structures of recombinant native and E202Q mutant human acetylcholinesterase complexed with the snake-venom toxin fasciculin-II. Acta Crystallogr D56: 1385-1394.

Lambert C, Leonard N, De Bolle X, Depiereux E (2002) ESyPred3D: Prediction of proteins 3D structures. Bioinformatics 18: 1250-1256.

Larkin M, Blackshields G, Brown N, Chenna R, McGettigan P, McWilliam H, Valentin F, Wallace I, Wilm A, Lopez R, Thompson J, Gibson T, Higgins D (2007) ClustalW and ClustalX version 2. Bioinformatics 23: 2947-2948.

Lenormand T, Bourguet D, Guillemaud T, Raymond M (1999) Tracking the evolution of insecticide resistance in the mosquito *Culex pipiens*. Nature 400: 861-864.

Lenormand T, Raymond M (2000) Clines with variable selection and variable migration: model and field studies. American Naturalist 155: 70-82.

Li F, Han, Z (2004) Mutations in acetylcholinesterase associated with insecticide resistance in the cotton aphid, *Aphis gossypii* Glover. Insect Biochem. Mol. Biol. 34: 397-405.

Mallender W, Szegletes T, Rosenberry T (2000) Acetylthiocholine binds to ASP74 at the peripheral site of human acetylcholinesterase as the first step in the catalytic pathway. Biochemistry 39: 7753-7763.

Millard C, Kryger G, Ordentlich A, Harel M, Raves M, Greenblatt H, Segall Y, Barak D, Shafferman A, Silman I, Sussman J (1999) Crystal structure of "aged" phosphylated *Torpedo* acetylcholinesterase: nerve agent reaction products at the atomic level. Biochemistry 38: 7032-7039.

Morel N, Bon S, Greenblatt H, Wodak S, Sussman J, Massoulié J, Silman I (1999) Effect of mutations in the peripheral anionic site on the stability of acetylcholinesterase. Mol. Pharmacol. 55: 982-992.

Munoz-Ruiz P, Rubio L, Garcia-Palomero E, Dorronsoro I, Monte-Milan M, Valenzuela R, Usan P, Austria C, Bartolini M, Andrisano V, Chanal AB, Orozco M, Luque FG, Medina M and Martinez A (2005) Design, synthesis and biological evaluation of dual binding acetylcholinesterase inhibitors: new disease-modifying agents for Alzheimer's disease. J. Med. Chem. 48: 7223-33.

Mutunga J, Anderson T, Carlier P, Bloomquist J (2010) Inhibition of Blattella germanica (German cockroach) Acetylcholinesterase by Tacrine dimers: Prospects for the molecular design of a selective insecticide for a household pest. In: Peterson C, Stout D (eds) Pesticides in Household, Structural and Residential Pest Management, American Chemical Society, Oxford University Press pp. 41-50.

Nabeshima T, Kozaki T, Tomita T, Kono Y (2003) An aminoacid substitution on the second acetylcholinesterase in the pirimicarb-resistant strains of the peach potato aphid, *Myzus persicae*. Biochem Biophys Res Commun 307:15-22.

Radic Z and Taylor P (2006) Structure and function of cholinesterases. In: Gupta R. (ed) Toxicology of organophosphate and carbamate compounds, Elsevier Academic Press, Amsterdam, pp 161-186.

Raves M, Harel M, Pang YP, Silman I, Kozikowski A, Sussman J (1997) 3D structure of acetylcholinesterase complexed with the nootropic alkaloid, (-)-huperzine A. Nat Struct. Biol. 4: 57-63.

Rydberg E, Brumshtein B, Greenblatt H, Wong D, Shaya D, Williams L, Carlier P, Pang YP, Silman, I and Sussman, JL (2006) Complexes of alkylene-linked tacrine dimers with Torpedo californica acetylcholinesterase: Binding of Bis 5- tacrine produces a dramatic rearrangement in the active-site gorge. *J. Med. Chem.* 49: 5491-5500.

Silver A (1974) Biology of Cholinesterases, pp. 72-73, American Elsevier Publishing Co., Inc., New York.

Summers WK, Tachiki KH, and Kling A (1989) Tacrine in the treatment of Alzheimer's disease. Europ. Neurol. 29(3): 28-32.

Sussman J, Harrel M, Frolow F, Oefner C, Goldman A, Toker L, Silman I (1991) Atomic structure of acetylcholinesterase from *Torpedo carlifornica*: a prototypic acetylcholine binding protein. Science 253: 872-879.

Tatusova T, Madden T (1999) Blast 2 sequences - a new tool for comparing protein and nucleotide sequences. FEMS Microbiol Lett. 174:247-250.

Toutant J (1989) Insect acetylcholinesterase: catalytic properties, tissue distribution and molecular forms. Prog Neurobiol 32:423-446.

Walker C (1983) Pesticides and birds-mechanisms of selective toxicity. Agric Ecosyst Environ 9: 211-226.

Wang H, Carlier P, Ho W, Wu D, Lee N, Li C, Pang YP, Han Y (1999) Effects of bis (7)-tacrine, a novel anti-Alzheimer's agent, on rat brain AChE. NeuroReport 10:789-793.

Weill M, Fort P, Berthomieu A, Dubois M, Pasteur N (2002) A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non-homologous to the ace gene in Drosophila. Proc R Soc Lond Ser B: Biol. Sci. 269: 2007-2016.

Wilson B, Samantha MK, Santhi K, Kumar KP, Ramasamy M and Suresh B (2009) Significant delivery of tacrine into the brain using magnetic chitosan microparticles for treating Alzheimer's disease. J. Neurosci. Methods. 177 (2): 427-433.

Zhang Y, Kua J and McCammon JA (2002) Role of the catalytic triad and oxyanion hole in acetylcholinesterase catalysis: An ab initio QM/MM study. J. Americ. Chem. Soc. 124: 10572-77.

Chapter 3. Toxicokinetics of Selective *N*-Methylcarbamates and Resistance Analysis of *An. gambiae* mosquitoes

Abstract

Toxicokinetics of previously reported mosquito-selective *N*-methylcarbamates were evaluated by topical and contact toxicity, synergism (piperonly butoxide and DEF) and cuticular penetration and compared to propoxur, a WHO standard. Experimental carbamates were synergized at greater levels than propoxur and P450-monooxygenase detoxification was the main mechanism, *in vivo*. Microinjection increased toxicity up to 22-fold in some chemicals, whereas thiolated compounds had no significant increase in toxicity. Several subclasses of monovalent and bivalent pyrazoles, oximes and sulfenylated compounds were assessed for toxicity to both susceptible (G3) and carbamate-resistant (Akron) mosquitoes. Unsubstituted acetaphenone oxime carbamates were toxic to both susceptible and resistant mosquitoes. However, substituted acetophenone and pyrazole or aliphatic oxime carbamates had little or no toxicity to mosquitoes. Monovalent pyrazole *N*-methylcarbamates showed superior contact toxicity to both susceptible and resistant mosquitoes, in a manner dependent upon side-chain branched substituents. *N*-sulfenylated analogs of toxic phenyl *N*-methylcarbamates were not toxic by contact assay, but were highly toxic when topically applied. Likewise, a bivalent phthalimide-pyrazole carbamate was toxic to mosquitoes by topical application and not by contact assay. Compounds described here are valuable leads to carbamate insecticides that are effective in controlling both susceptible and resistant mosquito populations.

3.1 Introduction

Chemical use of insecticides for mosquito control has remained an important tool and component for the integrated vector management of malaria and other vector-borne diseases. The Roll Back Malaria Abuja declaration in the year 2000 set forth an ambitious plan to reduce mortality and morbidity due to malaria in Africa through an array of ways and means, among them use insecticide treated nets (ITNs) and indoor residual spraying (IRS) coverage (WHO 2000). Since then, success in reducing malaria mortality and morbidity due to expansive ITN use has been reported in Africa (Amstrong *et al.* 2001, Diallo *et al.* 2004, Howard *et al.* 2003, Lengeler 2004, ter Kuile *et al.* 2004, Steketee *et al.* 2008, WHOPES 2010). Although IRS has also been widely used especially in malaria endemic areas, recent reports show that ITNs provide more superior protection against malaria (Pluess *et al.* 2010). ITNs also protect non-users through reduced mosquito survival, feeding success and feeding frequency, all leading to reduced density of vector populations (Hawley *et al.* 2003, Killeen *et al.* 2007, Geissbühler *et al.* 2007, Howard *et al.* 2000).

There are no new chemical insecticides developed for wide-scale public health use in the last 30 years, because of less investment by industry, compared to agricultural pesticides (Ranson *et al.* 2010). While pyrethroids are the only insecticides currently approved by WHO for use in ITNs (WHO, 1989; Zaim *et al.* 2000, Lengeler *et al.* 1996), continued use has led to the emergence of resistance, which is now widespread across Africa (See review by Ranson *et al.* 2010, and references therein). In recent semi-field studies, the use of carbamate-treated nets has shown greater efficacy in controlling mosquitoes, even in pyrethroid-resistant populations (Rowland and N'Guessan 2009, Guillet *et al.* 2000, Miller *et al.* 1991, Fanello *et al.* 1999, Kolaczinski *et al.* 2000, Curtis *et al.* 1998). However, these existing conventional carbamates,

though effective in killing mosquitoes, are highly toxic to humans (Guillet *et al.* 2000); thereby prohibiting their use. There is need to develop new insecticides that are less toxic to humans and effective on mosquitoes to be used as alternatives, in mixtures and/or rotations with pyrethroids.

Current commercial carbamates are not effective for control of *ace*-1 resistant mosquitoes, which express a modified acetylcholinesterase (MACE) due to a G119S mutation (Weill *et al.* 2004). It is therefore equally important not only to seek new and safer carbamate insecticides, but also those that mitigate resistance development. One proposed strategy to achieve this goal is to design bivalent ligands that are able to bind simultaneously and selectively to the peripheral and catalytic sites of mosquito AChE (Carlier *et al.* 2008; Pang *et al.* 2009; Zhao *et al.* 2008). In chapter one, I described the concept of bivalency, which was discovered through X-ray crystallography of *Tc*AChE (Sussman *et al.* 1991; Harel *et al.* 1993). In the past decade, highly potent bivalent AChE inhibitors have been developed as potential drugs for the management of Alzheimer's disease (Carlier *et al.* 2000; Pang *et al.* 1996, He *et al.* 2007, Feng *et al.* 2005). On the other hand, only a few insecticidal bivalent carbamates have been reported (Zhao *et al* 2008, Zhao *et al.*, 2009, Ma *et al.* 2010). These were not evaluated on mosquitoes, and neither were there reports of their selectivity to insect pests.

In this study, I investigated the efficacy of new *N*-methylcarbamates (Fig. 3.1) as candidate insecticides, in comparison to Propoxur (Baygon®) a WHO carbamate standard. Comparisons were made between residual contact toxicity on treated paper and intrinsic toxicity assessed by topical application. Synergism ratios were determined for the carbamates using piperonyl butoxide (PBO) to block *in vivo* detoxification by P450-monooygenases and also using DEF to block detoxification by carboxylesterases (Casida, 1970; Sanchez-Arroyo *et al*. 2001). Microinjections were performed to assess the role of the insect cuticle as a penetration barrier to

57

carbamates. Lastly, I assessed comparative toxicity of pyrazole and phenyl monovalent and

bivalent carbamates and their derivatives on susceptible and carbamate-resistant strains of *An.*

gambiae. Results presented here offer insights on the metabolic fate of these carbamates as well

as their efficacy in controlling carbamate-resistant mosquito populations, and guides future

improvement of existing carbamates.
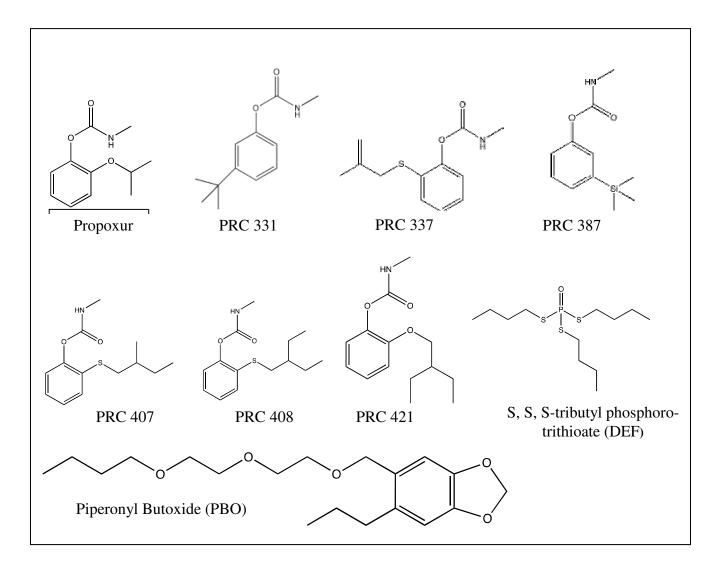
Figure 3. 1. Chemical structures of some *N*-methyl carbamates and synergists used in this study. PRC 331 and 387 are 3-substituted compounds whereas propoxur, PRC 337, 407, 408, and 421 are 2-substituted carbamates.

3.2 Materials and methods

3.2.1 Insects

An. gambiae G3 is a reference susceptible strain maintained at Virginia Tech. The Akron strain was obtained from the American Type Culture Collection (ATCC), through the CDC-MR4 (MR4-913, donated from Benin by Martin Akogbeto). Akron mosquitoes have both knockdown resistance (*kdr*) to pyrethroids (L1014F) and the G119S mutation (*ace*-1^R), which confers resistance to carbamates. Mosquito colonies are maintained at the Fralin Life Science Center insectary, at 75% relative humidity and 25 ^{o}C, with a 12/12 dark and light cycle. A walk-in environmental chamber in which bioassays were conducted was maintained under similar conditions.

3.2.2 Insecticidal carbamates, solvents and synergists

Experimental carbamates were synthesized and purified to > 95% purity in Dr. Paul Carlier's laboratory in the Department of Chemistry, Virginia Tech. Propoxur (technical grade), piperonyl butoxide (PBO, 90%), DEF, and ethanol were purchased from Sigma Chemical Co. (St. Louis, MO).

3.2.3 WHO paper toxicity assays

The WHO protocol (WHO, 1981) was used to assess contact toxicity of the chemicals on 2-5 day *An. gambiae* non-blood fed females, with some slight modifications. Mosquito toxicity data for propoxur and PRC 331 (Table 3.1) were obtained by Ania Wynsinski, a research associate in Dr. Sally Paulson's laboratory, Department of Entomology, Virginia Tech. A range

finding assay was performed with 1, 0.5 and 0.1 mg/mL single paper treatments after which a subsequent detailed assay followed to determine actual LC_{50} values. For each chemical, 5 concentrations giving 0-100% mortality were prepared and treated by applying 2 mL of each concentration on a 12 cm x 15 cm paper, with 95% ethanol as solvent. Mosquitoes were chilled for 3 min on ice, after which 25 females were placed in the WHO kit holding chamber (Fig. 3.2) to acclimatize for one hour. Mosquitoes were then moved to the treatment chamber wrapped on the inside with treated paper and left for 1 hr, after which they were transferred back to the holding chamber and maintained on 10 % sugar solution for 24 hrs. Each concentration was repeated in triplicate using different batches of mosquitoes to minimize inter-batch variability, with an ethanol-only treated negative control. Mortality was recorded 24 hr after treatment and in cases with control mortality ($< 20\%$), data was corrected using the Abbot's formula.

Abbot's formula:

$$\text{Corrected \% mortality} = \frac{\text{\% Alive in Control - \% Alive in Treatment}}{\text{\% Alive in the Control}}$$

Experiments having control mortality $\geq 20\%$ were discarded. Mortality data were analyzed by log-probit using Poloplus® (LeOra software Co. CA, USA) to generate the 24 hr LC_{50} of each chemical, the concentration at which 50% of treated mosquitoes were dead (Table 3.1).

Figure 3. 2. WHO assay kit for testing carbamate chemicals on adult mosquitoes. The holding and treatment (exposure) chambers are on the left and right; respectively.

3.2.4   Topical toxicity assays

The method of Pridgeon *et al* (2008) was used for topical application assays, with slight modifications. Briefly, mosquitoes were chilled on ice for 3 min, during which 200 nL of chemical (dissolved in 95% Ethanol) was applied onto the pronotum using a handheld Hamilton® microapplicator (Fig. 3.3). For each chemical, 5 doses were applied on 10 mosquitoes each, and repeated 3 times using different batches of mosquitoes. A solvent-only treatment was included in each experiment as a negative control. Mosquitoes were transferred into paper cups covered with netting and supplied with sugar water for 24 hours, after which mortality was recorded. Mortality data from the triplicate experiments was pooled and analyzed by log-probit using Poloplus® to determine 24 hr LD_{50} values, the dose at which killed 50% of treated subjects.

Figure 3. 3. A Hamilton® topical applicator fitted with a syringe.

3.2.5 *Synergism assays*

Synergism experiments were performed only for *N*-methylcarbamates shown in Fig. 3.1. Two different assays were performed in this study, a carbamate synergist co-treatment assay and a PBO-pretreatment assay. In the co-treatment experiments, a range of chemical concentrations were prepared each containing 1000 ng of PBO or DEF, and the treatment procedure was as described for topical assays. In synergist pre-treatment experiments; 200 ng of PBO was applied to mosquitoes 4 hrs before the chemical was topically applied. Previous reports indicate that PBO may act as a solvent in a co-treatment experiment and therefore generate pseudo-synergism effects (Sun and Johnson, 1972) by enhancing the penetration of the chemical through the cuticle. I conducted the 4 hr pre-treatment experiments in order to compare the data with co-treatment experiments and determine if there were differences between the two methods. Note that 1000 ng and 200 ng of synergist were used for co-treatment and 4-hr pretreatment experiments, respectively, as effective doses. These doses were determined by assessing the optimal amount of synergist that would have no lethal effects on the control experiments. Due to the fact that mosquitoes had to be chilled twice in ice during application of synergist and chemical (pre-treatment assays), the maximally tolerated PBO dose for pre-treatment experiment

was 5-fold lower than for co-treatment experiments. Mortality data for synergized and non-synergized experiments was collected 24 hr after insecticide exposure and analyzed by log-probit using Poloplus® to generate their respective LD_{50} values.

3.2.6 Microinjection studies

A manual injection system comprising a dissecting microscope, a syringe and fine glass needle was used to inject 0.2 μL into the ventral thoracic region of the mosquito. Based on observed toxicity with topical application studies, a suitable range of concentrations was prepared to inject nanogram quantities of chemicals that would generate 0-100% mortality; with a solvent-only set included as a negative control. Mortality data at 24 hr post-injection was analyzed by log-probit using Poloplus®, to determine the injected dose that kill 50% of the treated subjects (ID_{50}). I used the ratio of LD_{50} and ID_{50} to estimate the cuticular factor (CF), and used this as measure of cuticular barrier to penetration of the chemicals.

3.2.7 Ace-*1 genotyping in field collected and laboratory reared mosquitoes*

Adult mosquito sampling was done between June-July of 2007 in two different sites; namely, Western Kenya (Mbita-Kisumu), and Mwea, in central Kenya (Fig. 3.3). The resistance status of mosquitoes to pyrethroids in these areas had recently been characterized (Chen *et al*. 2008), but *ace*-1 resistance had not been evaluated. Up to 20 female indoor resting adult mosquitoes were collected by aspiration method from 10 randomly selected huts in each of the 2 sites. Samples were sorted to genus level using morphological keys (Coetzee *et al*. 1993) at the

field centers of the International Center of Insect Physiology and Ecology (ICIPE). Mosquitoes were preserved in 95% ethanol, and shipped to our laboratory for resistance genotyping.
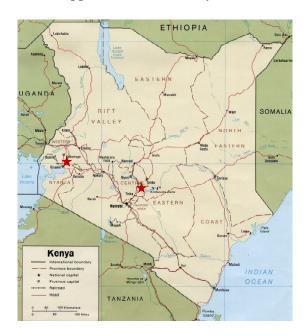
Figure 3. 4. Map of Kenya showing the two sites (red stars) where adult mosquito sampling was done. In each site, 10 huts were randomly selected and an average 20 adult mosquitoes collected from each hut.

Carbamate-resistance status for laboratory reared (G3 and Akron) and field-collected mosquitoes was evaluated using an allele specific PCR-RFLP method, as described by Weill *et al*. (2004), without modifications. Genomic DNA was extracted using a phenol-chloroform method by Scott *et al* (1993), suspended in DEPC water and stored at -20 $^{\circ}$C, awaiting further analysis. PCR-RFLP experiments were performed at the laboratory of Dr. Kevin Myles, in our department. The sequences of primers used are as shown below:

Forward primer (Moustdir1): 5' CCGGGNGCSACYATGTGGAA 3'

Reverse primer (Moustrev1): 5' ACGATMACGTTCTCYTCCGA 3.'

A 194 bp PCR fragment was amplified using these primers, in both *Anopheles gambiae* and *Culex* Sp. mosquitoes. The G119S mutation introduces a recognition site for the restriction enzyme, *Alu*1. After digestion of the PCR product with *Alu*-1, an *ace*-1 homozygous susceptible (SS) remained undigested (194 bp), a MACE homozygous resistant (RR) yielded two fragments of 74bp and 120bp, and an *ace*-1 heterozygote (RS) yields a combined pattern of 194 bp, 74 bp and 120 bp.

3.3 Results

3.3.1 *Toxicokinetics of* N-*methylcarbamates with* An. gambiae *G3 mosquitoes*

Toxicity of N-methylcarbamates to susceptible G3 strain of *An. gambiae* mosquitoes was assessed at four levels namely; contact toxicity (LC_{50}), topical toxicity (LD_{50}), synergized toxicity (synergism ratio-SR) and injected toxicity (ID_{50}).

Table 3. 1. Contact, topical, synergized, and injected toxicity of *N*-methylcarbamates to *An. gambiae* G3 mosquitoes.

Inhibitor	Contact toxicity LC_{50}, µg/mL	Topical toxicity, LD_{50}, ng/insect			Injected toxicity, ID_{50}, ng/insect
		No synergist	+PBO	+DEF	
Propoxur	37 (27-55)[a]	3 (2.4-4.2)[a,1]	2.1 (1.5-2.7)[a,1]	1.8 (1.4-2.4)[a,1]	0.24 (0.2-0.34)[a]
PRC 331	31 (14-60)[a]	4.5 (4-5)[a,1]	1.7 (1.4-2.0)[a,2]	1.8 (1.4-2.6)[a,b,2]	0.3 (0.17-0.5)[a]
PRC 337	69 (53-88)[b]	12.3 (11-13.4)[b,1]	3.4 (2.6-4.2)[b,2]	3.4 (2.5-4.4)[b,2]	11.2 (8.6-13.8)[b]
PRC 387	169 (162-176)[c]	8.0 (6.0-10)[c,1]	2.1 (1.2-3)[a,b,2]	6.7 (5-8.5)[c,1]	1.3 (0.92-1.7)[c]
PRC 407	1000 (70%)	11 (9.7-12.1)[b,1]	2.7 (2-3.5)[a,b,2]	3.4 (2.4-4.4)[b,2]	4.5 (3.6-5.4)[d]
PRC 408	1000 (27%)	10 (8.3-12.3)[b,c,1]	2.7 (1.8-3.6)[a,b,2]	3.3 (2.6-4)[b,2]	12.1 (10.3-14)[b]
PRC 421	1000 (27%)	81 (64-95)[d,1]	18 (16-20)[c,2]	38 (33-43)[d,3]	3.7 (3-4.5)[d]

Values in brackets indicate 95% CI or percent mortality at the highest concentration tested. Values with same superscript letters or numbers in the same column or row, respectively are statistically not significant, based on overlapping 95% CI ($P < 0.05$). Synergism ratios and cuticular factors (CF) for these carbamates are shown in Figure 3.3.
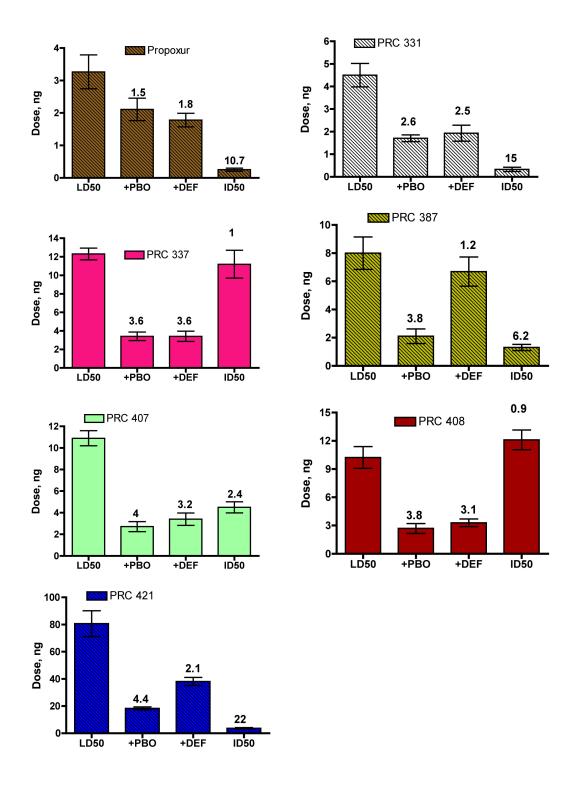
Figure 3. 5. Topical, synergized and injected toxicity of *N*-methyl carbamates to G3 mosquitoes. Numbers above PBO and DEF bars represent a synergistic ratio (SR = LD_{50} of insecticide alone / LD_{50} of insecticide + synergist), whereas those above ID_{50} bars are cuticular factors computed from the LD_{50}/ID_{50} ratios. Error bars represent 95% confidence intervals.

67

Synergism by PBO and DEF led to an overall increase in toxicity, though at varying levels across compounds. Propoxur and PRC 331 are statistically equitoxic, whether by contact, topical, synergized or injection assays (Table 3.1). Except for Propoxur (SR=1.5; Fig. 3.5), synergism by PBO was statistically higher in all other compounds, when compared with non-synergized toxicity. On the other hand, synergism by DEF was statistically significant in all compounds except propoxur and PRC 387 (Table 3.1). Synergism by PBO was highest with PRC 421 (SR= 4.4) and lowest in propoxur (SR= 1.5). Synergism by DEF was highest with PRC 337 (SR= 3.6) and lowest with PRC 387 (SR= 1.2). Overall, PBO synergism was greater than synergism by DEF. The gain in toxicity by injection was highest in PRC 421 (CF= 22), followed by PRC 331, propoxur, PRC 387, 407, 337 and 408, in descending order. Gain in injected toxicity was lowest in sulfur containing compounds (i.e.) PRCs 337, 407 and 408.

In order to compare the effects of PBO co-treatment and 4-hr pretreatment (PtSR) on the toxicity of *N*-methylcarbamates to mosquitoes, I computed their respective synergism ratios (CoSR and PtSR), as summarized in Table 3.2. Based on the SR values, I determined synergism differential index (SDI), where SDI value > 1 indicates that synergism was greater with 4-hr pretreatment than in co-treatment experiments, and vice-versa. Only PRC 331 (SDI= 3.1) showed appreciable gain in PBO-synergism with 4hr pretreatment experiments compared to co-treatment experiments. All other compounds showed approximately 1.5-fold difference or less, between SR values obtained with the two methods.

Table 3. 2. PBO co-treatment and 4-hr pretreatment synergism ratios (SR) for *N*-methylcarbamates with G3 mosquitoes.

Inhibitor	CoSR	PtSR	SDI
Propoxur	1.5	2.3	1.5
PRC 331	2.6	8	3.1
PRC 337	3.6	4	1.1
PRC 387	3.8	3.3	0.87
PRC 407	4.0	3.8	0.95
PRC 408	3.8	2.5	0.66
PRC 421	4.4	6.2	1.4

SR are LD_{50} without synergist/LD_{50} with synergist, with CoSR obtained from co-treatment experiments and PtSR obtained by 4-hr PBO pretreatment experiments. SDI = PtSR/CoSR.

3.3.2 Ace-*1 genotyping in field collected and laboratory reared mosquitoes*

The carbamate-resistance status of laboratory reared (G3 and Akron strains) and field-collected *Anopheles* and *Culex* mosquitoes was evaluated. A representative agarose gel showing the different samples tested is shown in Fig. 3.6. A total of 200 *Anopheles* sp. and 50 *Culex* sp. field collected mosquitoes were assayed. From this data, the G3 strain and the field-collected *An. gambiae* are SS, the Akron strain is RR, and the field-collected *Culex* sp. mosquito is RS. There was 100% fidelity of genotypes in SS G3 and RR Akron mosquitoes, as predicted from the primers used. No *wild Anopheles gambiae* showed the resistant phenotype of *ace-1* (n = *ca*. 200)*, whereas a single wild *Culex* mosquito from Kenya was heterozygous for MACE resistance was observed (Fig. 3.6).
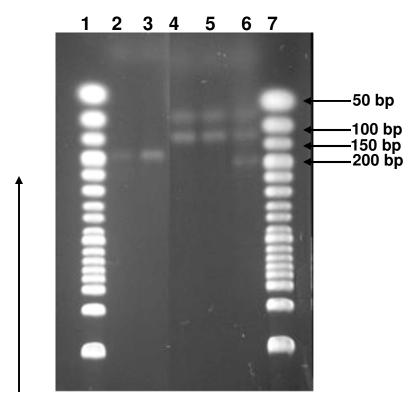
Figure 3. 6. Agarose gel showing resolved PCR-RFLP products of *Ace*-1 gene in susceptible (G3), resistant (AKRON) *An. gambiae*, and field collected mosquito samples. Lanes 1 and 7 are 50 bp ladders, lane 2 is *An. gambiae* collected in Kenya, and lane 3 is a G3 insecticide susceptible *An. gambiae*. Lanes 4 and 5 are lab-reared *ace*-1 resistant F21 Akron *An. gambiae* mosquitoes, and Lane 6 is a field-collected *Culex* spp. mosquito. The vertical arrow shows direction of electrophoresis.

3.3.3 Toxicity of conventional and experimental N-*methylcarbamates to susceptible (G3) and carbamate-resistant (Akron)* An. gambiae *mosquitoes*

Table 3.3 summarizes the toxicity, and resistance ratio of some selected commercial carbamates, and related experimental *N*-methyl carbamates to the susceptible G3 strain and the resistant Akron strain.

Aldicarb and methomyl are aliphatic oxime carbamates. PRCs 331, 521 and 608 are *meta* substituted alkyl phenyl N-methylcarbamates, whereas propoxur and PRC 431 have their branched ether substituents at the *ortho* position. The branched phenyl ring substituents are at positions that historically confer maximal AChE inhibition potency and toxicity (Metcalf, 1965,

70

Fukuto, 1967). Some compounds were tested either topically or by contact assay; consequently

LC_{50} or LD_{50} values were used to assess the resistance ratio (RR), where applicable (Table 3.3).

Table 3. 3. Selectivity and toxicity of commercial and experimental *N*-methylcarbamates.

Carbamate	[a]G3 LC_{50} or LD_{50}	[a]Akron LC_{50} or LD_{50}	(RR)[b]
Propoxur	37 µg/mL	0% @ 5000 µg/mL	>135
Aldicarb	70 µg/mL	33 µg/mL	0.5
Methomyl	24 µg/mL	0% @ 5000 µg/mL	-
Bendiocarb	15 µg/mL	0% @ 5000 µg/mL	-
PRC 331	4.5 ng	348 ng	77
PRC 521	1.6 ng	27 ng	17
PRC 608	41 µg/mL	716 µg/mL	17
PRC 431	290 µg/mL	0% @ 1000 µg/mL	-

[a]LC_{50} values are µg/mL, using the WHO insecticide-treated filter paper assay, and topical LD_{50} values are ng/insect. [b]RR denotes resistance ratio, determined by dividing LC_{50} or LD_{50} values of the Akron strain by those of the G3 strain. An RR value of 1 means the carbamate is equitoxic toxic to both strains; RR > 1 means the toxicity in higher in the susceptible G3 mosquitoes; if RR < 1 indicates that toxicity is greater in the Akron resistant mosquitoes.

The phenyl *N*-methylcarbamates showed a variable level of toxicity to both mosquito

strains. For example, both PRC 521 and 608 were toxic to G3 and Akron mosquitoes, compared

to PRC 331. Propoxur and PRC 431 were toxic to G3 but not Akron mosquitoes. Additionally,

PRC 431 was the most selective whereas PRC 521 was the least selective. Bendiocarb was the

most toxic commercial carbamate to G3, yet non-toxic to Akron mosquitoes (Table 3.3).

Aldicarb showed negative cross resistance between the *An. gambiae* strains, with ca. 2-fold

higher toxicity to the resistant strain that the susceptible G3. Methomyl, an oxime structurally

related to aldicarb (Fig. 3.7) was more toxic to G3 than aldicarb, but surprisingly nontoxic to

Akron mosquitoes.
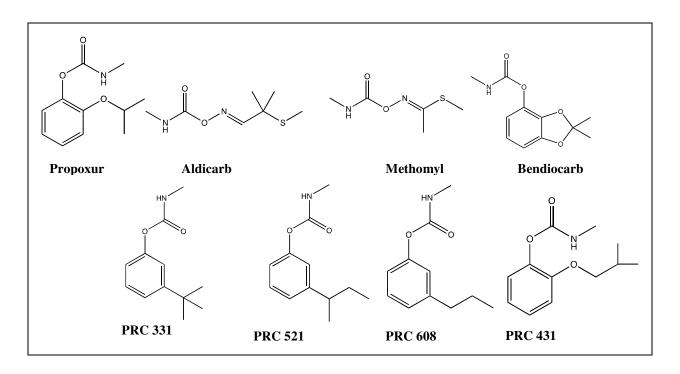
Figure 3. 7. Structures of conventional and experimental *N*-methylcarbamates, whose mosquito toxicity is shown in the table 3.3.

3.3.4 Toxicity of trifluorinated acetophenone and pyrazole oxime carbamates to An. gambiae *susceptible (G3) and resistant (Akron) mosquitoes*
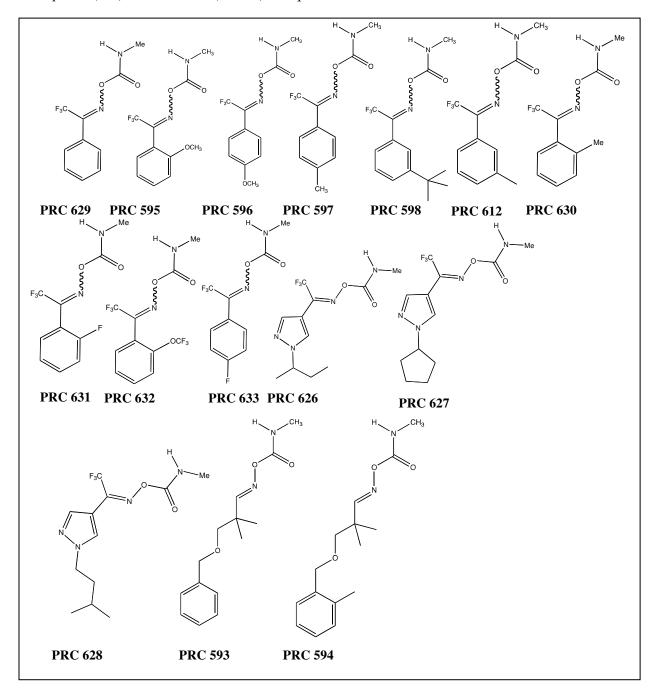
Figure 3. 8. Chemical structures of acetophenone and pyrazole oxime carbamates.

Table 3. 4. Toxicity of acetophenone and pyrazole oxime *N*-methyl carbamates to G3 and Akron mosquitoes.

Compound	G3 Toxicity[*]	Akron Toxicity[*]	RR[#]
PRC 629	91 (87-96)[a]	332 (309-353)[a]	4
PRC 595	131 (116-145)[b]	643 (614-674)[b]	5
PRC 596	0% @ 1 mg/mL	NT	-
PRC 597	320 (315-345)[c]	ND	-
PRC 598	0% @ 1 mg/mL	NT	-
PRC 612	100% @ 1mg/mL	0% @ 1mg/mL	-
PRC 630	131 (120-145)[b]	242 (186-316)[a]	1.8
PRC 631	314 (284-345)[c]	693 (653-733)[b]	2.2
PRC 632	457 (438-475)[d]	939 (882-1061)[c]	2.1
PRC 633	334 (300-365)[c]	580 (554-605)[d]	1.7
PRC 626	50% @ 0.5 mg/mL	0% @ 1mg/mL	-
PRC 627	40% @ 1mg/mL	NT	-
PRC 628	40% @ 1mg/mL	NT	-
PRC 593	0% @ 1 mg/mL	NT	-
PRC 594	8% @ 1 mg/mL	NT	-

LC_{50} values (µg/mL) in the same column and bearing the same superscript letter are not significantly different based on their overlapping 95% CI. [*]Toxicity determined using the WHO treated filter paper assay. Percentages represent mortality at the tested carbamate concentration. ND denotes "not done" meaning that the experiment is pending, whereas NT denotes not tested, usually because the compound was of low toxicity to the susceptible G3 strain. [#]RR is the resistance ratio, computed by dividing Akron LC_{50} by G3 LC_{50}.

The trifluorinated un-substituted acetophenyloxime carbamate (PRC 629) was the most toxic to G3 for this series, and was used as a reference compound upon which side-chain structural modifications were made (Fig. 3.8 and Table 3.4). Both PRC 629 and PRC 630 (methyl substituted at *ortho*) were the most toxic compounds to Akron, at levels that are statistically similar. Substitution with a methoxy at the *ortho* position (PRC 595) caused a slight decline (< 2-fold) in mosquito toxicity, whereas substitution at the *para* position (PRC 596) abolished mosquito toxicity. Comparatively, a methyl substitution at *para* (PRC 597) is tolerated better than an analogous methoxy substitution (PRC 596). Although *ortho* methoxy (PRC 595) and *ortho* methyl (PRC 630) were equitoxic to G3 mosquitoes, the methyl analog was

ca. 3-fold more toxic to Akron mosquitoes, indicative of a better fit of the smaller methyl group in to the crowded G119S AChE active site. Fluorination of the phenyl ring or its methyl substituents (PRC 631, PRC 632 and 633) was unfavorable due to observed decrease in toxicity to both strains of mosquitoes. Although the actual G3 LC_{50} value for PRC 612 (*meta*-methyl) and Akron LC_{50} value for PRC 597 (*para*-methyl) were not determined, mosquito toxicity data shows that a methyl substitution at *ortho* (PRC 630) was the most favorable. Extended branching of PRC 612 to make a *tert*-butyl substituent (PRC 598) led to complete loss of toxicity. In the course of synthesizing carbamate analogs, allophanate by-products, such as the pyrazoles (PRCs 626-8) were observed, and it was deemed necessary to screen them for activity. The allophanate pyrazoles turned out to be inactive, and similarly a pair of aldicarb oxime analogs that were prepared (PRCs 593-4) showed no toxicity to mosquitoes.

3.3.5 Toxicity of pyrazole carbamates to susceptible and resistant Anopheles gambiae *mosquitoes*
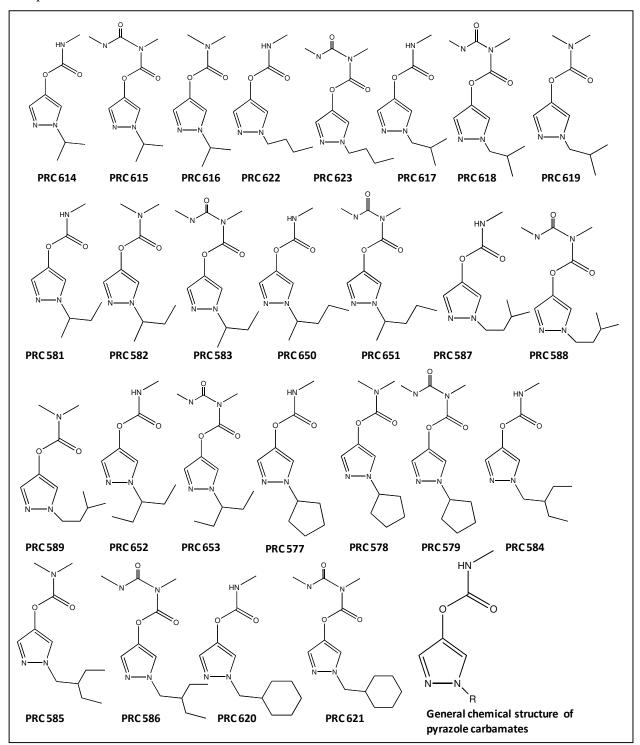
Figure 3. 9. Structures of allophanates, *N*, *N*-dimethyl, and *N*-methyl pyrazole carbamates.

Table 3. 5. Toxicity of allophanates, *N*, *N*-dimethyl, and *N*-methyl pyrazole carbamates to susceptible and resistant mosquitoes.

Compound	R*	G3 Toxicity[a]	Akron Toxicity[a]	(RR)[b]
PRC 614	*iso*-propyl	30% @ 0.1 mg/mL	30% @ 0.5 mg/mL	-
PRC 615	allophanate-PRC 614	0% @ 1 mg/mL	NT	-
PRC 616	DM analog-PRC 614	0% @ 1 mg/mL	NT	-
PRC 622	*n*-butyl	50% @ 1 mg/mL	NT	-
PRC 623	allophanate-PRC 622	20% @ 1 mg/mL	NT	-
PRC 617	*iso*-butyl	41 (38-44)	10% @ 1mg/mL	-
PRC 618	allophanate-PRC 617	20% @ 1 mg/mL	0% @ 1 mg/mL	-
PRC 619	DM analog-PRC 617	30% @ 1 mg/mL	NT	-
PRC 581	*sec*-butyl	60 (56-64)	11 (10-13)	0.18
PRC 582	DM analog-PRC 581	124 (117-132)	202 (187-217)	1.6
PRC 583	allophanate-PRC 581	8% @ 1 mg/mL	NT	-
PRC 650	Pentan-2-yl	154 (140-167)	267 (241-289)	1.7
PRC 651	allophanate-PRC 650	60% @ 1 mg/mL	30% @ 1mg/mL	-
PRC 587	*iso*-pentyl	269 (253-283)	948 (887-1103)	3.5
PRC 588	allophanate-PRC 587	16% @ 1 mg/mL	NT	-
PRC 589	DM analog-PRC 587	44% @ 1 mg/mL	90% @ 1 mg/mL	-
PRC 652	Pentan-3-yl	138 (126-151)	231 (217-245)	1.7
PRC 653	allophanate-PRC 652	0% @ 1 mg/mL	40% @ 1mg/mL	-
PRC 577	cyclopentyl	29 (26-32)	365 (344-384)	13
PRC 578	DM analog-PRC 577	36% @ 1 mg/mL	NT	-
PRC 579	allophanate-PRC 577	48% @ 1 mg/mL	NT	-
PRC 584	2-ethylbutyl	20% @ 1 mg/mL	NT	-
PRC 585	DM analog-PRC 584	48% @ 1 mg/mL	NT	-
PRC 586	allophanate-PRC 584	60% @ 1 mg/mL	NT	-
PRC 620	cyclohexylmethyl	30% @ 1 mg/mL	NT	-
PRC 621	allophanate-PRC 620	20% @ 1 mg/mL	NT	-

[a]Toxicity determined using the WHO treated filter paper assay. Values are LC_{50}s, in μg/mL and their 95% CI are shown in brackets, unless otherwise stated. Percentages represent mortality at the tested carbamate concentration, and statistical significance was assessed based on overlapping 95% CI. ND denotes 'not done,' meaning that experiment is pending, whereas NT denotes not tested, usually because the compound was of low toxicity to susceptible G3 strain.
[b]RR is the resistance ratio, computed by dividing Akron LC_{50} by G3 LC_{50}. *DM in this column denotes an N'N' dimethyl derivative.

Pyrazole *N*-methylcarbamates together with their allophanate and *N*, *N*-dimethyl derivatives were alkyl substituted at the 1-position, as shown in the general formula (Fig. 3.9, lower right hand corner). PRC 614, an *iso*-propyl substituted pyrazole was appreciably toxic to both G3 (30% dead at 0.1 mg/mL) and Akron mosquitoes (30% dead at 0.5 mg/mL). The allophanate and *N'N'*-dimethyl derivatives of PRC 614 (PRCs 615, 616) were both nontoxic to mosquitoes. PRC 622 is an *n*-butyl analog of PRC 614, and it was nontoxic to mosquitoes. Extending the side-chain by a methyl to make a *sec*-butyl substituent (PRC 581) led to a dramatic increase in toxicity to both strains of mosquitoes. The toxicity of PRC 581 to Akron mosquitoes was ca. 5.5 times greater than in G3, and bears one of the lowest RR value (0.18) observed so far in this study. Unlike PRC 614, the *N*, *N*-dimethyl derivative of PRC 581 (PRCs 582) was toxic to both mosquito strains, although at levels 2-5 fold lower than PRC 581. PRC 617 bears an *iso*-butyl substituent and compared to the *sec*-butyl isomer (PRC 581), PRC 617 was slightly more toxic to G3 but nontoxic to Akron mosquitoes. The allophanate (PRC 618) and *N*, *N*-dimethyl (PRC 619) derivatives of PRC 617 were nontoxic to mosquitoes.

Extended branching by a methyl group to form an *iso*-pentyl substituent (PRC 587) caused a ca. 4-7 fold loss in G3 toxicity, and a nearly complete loss of Akron toxicity, when compared to the *iso*/*sec*-butyl substituents. The isomeric penta-2-yl substituted analog (PRC 650) was ca. 2-fold more toxic to G3 and 4-fold more toxic to Akron mosquitoes, compared to PRC 587. PRC 652 is a pentan-3-yl side-chain branched isomer of PRC 587, and its toxicity to both mosquito strains was similar to that of PRC 650, the penta-2-yl isomer. A cylopentyl isomer (PRC 577) was the most toxic to G3 mosquitoes in this series, representing 4- and 9-fold higher toxicity than PRC 652 and 587; respectively. Although statistically different, the Akron toxicity of PRC 577 was comparable to that of isomeric PRC 650 and 652. The *N*, *N*-dimethyl

(PRC 578) and allophanate (PRC 579) analogs of PRC 577 had very low toxicity to mosquitoes. By further extending the side-chain to make an ethylbutyl substituent (6 carbon chain; PRC 584), the excellent mosquito toxicity observed with 5 carbon chain analogs was completely abolished. A methyl-cyclohexyl substituted analog (7 carbon chain, PRC 620) was also nontoxic to mosquitoes.

3.3.6 Toxicity of sulfenylated N-methylcarbamates to mosquitoes

Sulfenylated *N*-methylcarbamates were also tested because these compounds have been shown to have lower mammalian toxicity, as described and referenced in the discussion.

Figure 3. 10. Chemical structures of sulfenylated analogs of PRC 331 and 387.

Among the sulfenylated compounds, only PRC 654 and 657 showed marginal contact toxicity to mosquitoes. PRC 654 killed 70% of G3 mosquitoes in 24 hrs at 0.5 mg/mL, but was not toxic at 0.1 mg/mL. Likewise, PRC 657 had 100% G3 mortality in 24 hrs at 1 mg/mL but was not toxic at 0.5 mg/mL. Despite showing no contact toxicity, PRC 655 was highly toxic to G3 mosquitoes when topically applied (LD_{50} = 2 ng). Topical application data with G3 are incomplete; neither do I have Akron data at this point. When available, these data will help understand the SAR of this promising series of compounds in detail.

Table 3. 6. Toxicity of sulfenylated *N*-methylcarbamates to G3 *An. gambiae*.

Compound	Contact Toxicity[a]	Topical Toxicity[b]
PRC 654	0% @ 1 mg/mL	NT
PRC 655[*]	70% @0.5 mg/mL	2.0 ng (1.8-2.3)
PRC 656	0% @ 1 mg/mL	NT
PRC 657	100% @ 1 mg/mL	ND
PRC 658	0% @ 1 mg/mL	NT

[a]Toxicity at 24 hrs determined by WHO treated filter paper assay and [b]topical application assay. "ND" denotes 'not done,' meaning that experiment is pending, whereas "NT" denotes not tested, usually because the compound was of low toxicity to the susceptible G3 strain.

Chemical synthetic efforts were also directed towards the construction of bivalent inhibitors that might occupy both the peripheral and catalytic sites on the mosquito enzyme. The compounds utilized a pyrazole as the central moiety (Fig. 3.11), because of its smaller size relative to the phenyl ring that gave high cross-resistance values. These were tethered to a pthalimido group via alkyl tether (Fig. 3.11). The bivalents tested showed low contact toxicity to mosquitoes in the WHO paper assay (Table 3.7), regardless of changes in tether length, or *N*, *N*-dimethyl and allophanate derivatives. However, topical assays of PRC 590 with G3 and Akron mosquitoes gave the following results: G3 LD_{50} = 63 ng (55-72) and Akron LD_{50} = 386 ng (340-430), resulting in a RR = 6. Topical application data indicates that the bivalent compound is toxic to both mosquito strains and therefore an ideal candidate to use in designing more related compounds.

3.3.7   Toxicity of experimental bivalent carbamates to susceptible G3 mosquitoes
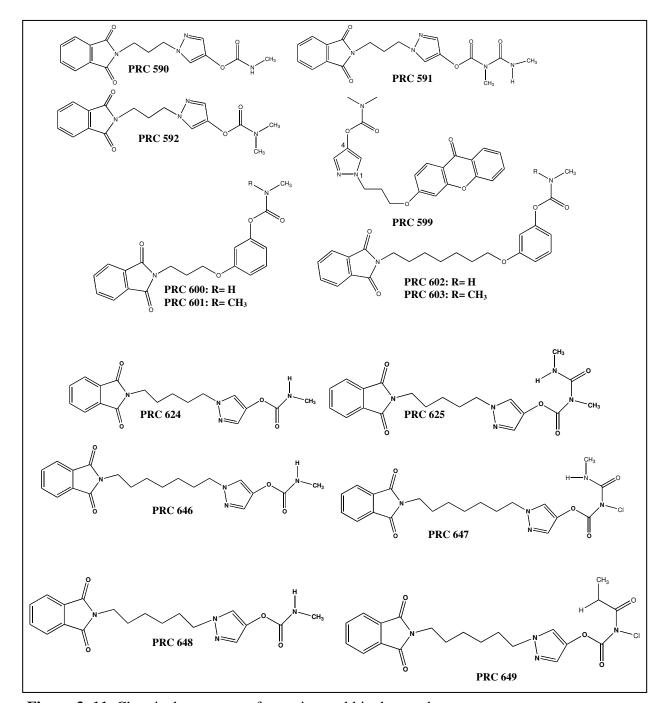
Figure 3. 11. Chemical structures of experimental bivalent carbamates

Table 3. 7. Toxicity of bivalent carbamates to G3 mosquitoes.

Compound	G3 Contact Toxicity[a]	Compound	G3 Contact Toxicity[a]
PRC 590	16% @ 1 mg/mL	PRC 603	0% @ 1 mg/mL
PRC 591	28% @ 1mg/mL	PRC 624	20% @ 1 mg/mL
PRC 592	12% @ 1 mg/mL	PRC 625	0% @ 1 mg/mL
PRC 599	0% @ 1 mg/mL	PRC 646	10% @ 1 mg/mL
PRC 600	0% @ 1 mg/mL	PRC 647	10% @ 1 mg/mL
PRC 601	0% @ 1 mg/mL	PRC 648	0% @ 1 mg/mL
PRC 602	0% @ 1 mg/mL	PRC 649	0% @ 1 mg/mL

[a]Toxicity determined by WHO treated filter paper assay and mortality assessed at 24 hr post-treatment.

3.4 Discussion

Current registered carbamates such as propoxur, bendiocarb and aldicarb pose a toxicological risk to humans due to low selectivity and hence cannot be used in insecticide treated nets as substitutes to or rotations with pyrethroids.   In this study, I have tested several chemical subclasses of carbamates that might be used for vector control, and their penetration and uptake properties, at least as reflected by toxicity resulting from various routes of exposure.

3.4.1   Toxicokinetics of N-*methylcarbamates: studies on topical and contact toxicity, synergism and cuticular penetration*

Previous reports from our group showed a series of *N*-methylcarbamates, which were highly selective to mosquito acetylcholinesterase (Carlier *et al*. 2008). PRC 331 was shown to be more a more potent inhibitor of mosquito AChE (Carlier *et al*. 2008) than propoxur, a WHO

standard. This compound (3-*tert*-buytlphenylmethylcarbamate) was identified years ago by its high enzyme inhibitory potency and toxicity to houseflies (Metcalf R.L., 1951), and also its toxicity (LD_{50} = 1.9 ng/female) to *Anopheles stephensi* (Hadaway and Barlow, 1965). In my studies, PRC331 and propoxur were statistically equitoxic at contact, topical, synergized or injected toxicity (Table 3.1), suggesting that differences in metabolism might be responsible. Despite low contact toxicity observed with some of the compounds (e.g., PRCs 407 and 408), their topical toxicity was comparable to that of contact-active carbamates, such as PRC 337 and 387. This finding implies that topical assays unmasked possible loss of toxicity associated with contact assays, perhaps via poor transfer from the paper.

All compounds were synergized at statistically significant levels, except propoxur. Greater sensitivity of PRC 331 to oxidative metabolism than propoxur would explain why it is not as toxic, *in vivo*, as expected from its superior enzyme potency. Comparison of synergism between isosteres PRC 331 and 387 shows significant differences in contact, topical, and DEF-synergized toxicity. However, the two compounds are statistically equitoxic when synergized by PBO, indicating that detoxication by P450-monooxygenases is higher in silicon-containing compounds than in their carbon analogs. The silyl carbamate, PRC 387, was also significantly synergized by PBO (ca. 4-fold), interestingly; synergism by DEF was not statistically significant. Similar to observations with propoxur and PRC 331, synergism of PRC 407 and 408 by both PBO and DEF was equal. Recent reports indicate that PBO can inhibit both P450 mono-oxygenases and carboxylesterases (Khot *et al.* 2008), which for some compounds would explain the equal SRs for PBO and DEF. Finally, only PRC 331 showed significantly greater synergism from four hr pretreatment, which is odd, in that it certainly did not have the highest SR. Perhaps there was an unusual effect on penetration of this compound.

Both PRC 408 and 421 are *S*- and *O*- containing analogs with identical side chains, whose estimated contact toxicity to mosquitoes was similar (both kill 27% at 1 mg/mL; Table 3.1). However, topical toxicity of PRC 408 was *ca*. 8-fold higher than that of PRC 421. The two compounds display a similar patter in the degree of synergized toxicity. Sulfur-containing compounds can be bioactivated to their more toxic sulfoxide and sulfone derivatives, *in vivo* (Casida *et al*. 1974, Risher, 1987). The up to 4-fold PBO synergism of PRC 337, 407 and 408 indicate that *in vivo* microsomal bioactivation was unlikely or it may have occurred through documented non-P450-mediated extramicrosomal system in mosquitoes (Ohkawa and Casida, 1971).

Microinjection studies showed increased levels of toxicity, except for the *S*-containing compounds. Lack of gain in toxicity by injection observed with these compounds (PRCs 337, 407 and 408) was a puzzling and unexpected observation. It is possible that *S*-containing carbamates possess excellent cuticular penetration and therefore their toxicity remains largely unchanged whether they are injected or topically applied. The second possibility is that despite their increased *in vivo* availability when injected, these compounds may be sequestered or have reduced penetration of the blood brain barrier (BBB) in the insect CNS. Either would translate to no gain in toxicity regardless of the level of their bioavailability in the insect hemolymph.

3.4.2 Toxicity of conventional and experimental phenyl and oxime N-*methylcarbamates to susceptible and carbamate resistant mosquitoes*

The search for mosquito selective carbamates should also address the existing problem of carbamate resistance, and the likelihood of selection for resistance. The genotype of the G3 (susceptible) and Akron (resistant) reference *An. gambiae* strains used in this study was validated

84

by PCR-FLP of the *ace*-1 gene (Weill *et al.* 2004). Only one *Culex* was found to possess the MACE mutation in about 200 sampled, suggesting that this mutation in AChE is not widely distributed in the areas of Kenya that I sampled.

Just like propoxur, PRC 331 was not toxic to Akron mosquitoes by contact toxicity assay, but PRC 331 did have measurable activity against Akron mosquitoes when topically applied. Although the toxicity of PRC 331 to Akron mosquitoes was subtle, it was unexpected given its similarity in pharmacological and toxicological properties to propoxur. Interestingly, both a *sec*-butyl isomer (PRC 521) and an *n*-propyl analog (PRC 608) were toxic to susceptible and resistant mosquitoes. This series of compounds shows that appropriate alkyl-substituted phenyl *N*-methylcarbamates are able to bind to the crowded G119S site in mosquito AChE, unlike their alkoxy-substituted counterparts. The greater toxicity of aldicarb to Akron mosquitoes is also probably due to better fit into the active site, and perhaps enhanced bioactivation to more toxic sulfone and sulfoxide derivatives *in vivo*. This is a likely scenario considering the fact that Akron mosquitoes possess both *ace*-1 and pyrethroid (*kdr*) resistance, the latter of which is often associated with elevated P450-monooxygenase activity (Chen *et al.* 2008, Hemingway *et al.* 2004; Karunaratne *et al.* 2007). Lack of Akron toxicity with methomyl, which is structurally related to aldicarb, remains a subject of further investigation, but may be due to the methyl group on the oxime carbon causing a structural clash with an amino acid of the enzyme catalytic site.

3.4.3 Toxicity of experimental trifluorinated acetophenone and pyrazole oxime carbamates to susceptible and carbamate resistant mosquitoes

Trifluorinated acetophenones have been shown to have high affinity to HuAChE (Piazzi *et al.* 2003) and therefore their carbamate-derivatives were evaluated for insecticidal activity in

mosquitoes. Un-substituted phenyl ring (PRC 629) or *ortho* position substituents (PRC 595, 596) were structurally preferred for toxicity of these compounds, whereas *meta* groups were of low activity. By substituting the *para*-methoxy of PRC 596 with a *para*-methyl group (PRC 597), appreciable G3 toxicity was observed, suggesting that not only the position on the phenyl ring but also the size of the substituent influences toxicity. Extended alkyl branching at the *meta* position (*tert*-butyl group, PRC 598) resulted in complete loss of toxicity to mosquitoes. Since extended alkyl branching was not tested at other positions, it is uncertain whether the same effect will hold true at *para* and *ortho* positions. Fluorination of the phenyl ring at *ortho* (PRC 631) or *para* positions (PRC 633) had little effect, and neither did trifluorination of the *ortho* methoxy substitutent (PRC 632). Presumably, the desired fluorine-mediated protection of the phenyl side-chain and its substituents, from metabolic detoxification was therefore not achieved. However, it is also possible that fluorinated analogs are poor AChE inhibitors but this cannot be confirmed since AChE inhibition data was yet to be obtained. To some extent, observations with positional substitution at the phenyl ring of acetophenone oxime carbamates agree with previous findings that *ortho* alkoxy and *meta* halo-, alkyl or aryl substitutions are favorable for insecticidal activity (Metcalf and Fukuto, 1965; Fukuto *et al*. 1967, Hadaway, 1971). Despite the loss in toxicity observed with phenyl substituents, some compounds in this series showed RR values near unity (PRC 630-633; table 3.4). It is therefore a promising subclass worth of further investigation to evaluate their selectivity and scale up mosquito toxicity.

Attempts to make toxic pyrazole oxime derivatives from toxic monovalent pyrazole *N*-methylcarbamates were futile. Note that PRC 626, 627 and 628 are trifluorinated oxime analogs of PRC 581, 577 and 587 pyrazole compounds, the latter group is toxic to both Akron and G3 mosquitoes (Table 3.5). Likewise, extended phenyl derivatives of aldicarb (PRC 593 and 594)

were nontoxic to mosquitoes, suggesting this series of structural modifications should be abandoned.

3.4.4 Toxicity of side-chain branched pyrazole N-*methylcarbamates is optimal at* sec-*butyl and varies at extended alkyl substituents*

A series of alkyl side chain elongation and branching at the 1-position were conducted to assess their effects on mosquito toxicity. Modest to high activity was typical for small alkyl substituents in this series. The *sec*-butyl isomer (PRC 581) was toxic to both strains of mosquitoes, and like aldicarb, showed negative cross-resistance to Akron. However, since G3 toxicity of PRC 581 declined compared to PRC331, maximal toxicity to both mosquito strains may require different spatial configurations of the inhibitor at the AChE active site. Further extension of the side-chain generated pentyl-substituted analogs with appreciable activity and relatively low RRs.   Larger or branched analogs of these compounds (e.g., PRC 584) led to a complete loss of toxicity to both strains of mosquitoes, and therefore they are probably too large to fit into the active site of AChE. .

For most of the pyrazole compounds discussed so far, an allophanate and *N, N*-dimethyl derivative was also tested. It was hypothesized that hydrolysis of the allophanate or *N*-demethylation would liberate an active carbamate, *in vivo*, and therefore these compounds were considered propesticidal. Only PRC 582, an *N, N*-dimethyl analog of PRC 581 was toxic to both mosquito strains, but of ca. 2-fold and 20-fold decreased toxicity to G3 and Akron mosquitoes, respectively, compared to the parental compound (PRC 581). Since PRC 581 was very toxic to mosquitoes, it is possible that limited demethylation, *in vivo*, generated small quantities of this

compound. Overall, these findings concur with previous findings (Metcalf, 1971) that *N*-demethylation is not a major detoxification pathway for *N*-methylcarbamates.

3.4.5 Toxicity of sulfenylated and bivalent N-*methylcarbamates and their analogs, to susceptible and carbamate-resistant mosquitoes*

N-arylsulfenyl and *N*-alkylsulfenyl derivatives of methylcarbamate insecticides were shown to have lower mammalian toxicity and more effective mosquito larvicides than the parent methylcarbamates (Black *et al.* 1973). Black *et al.* (1973) also reported that the selective toxicity does not relate directly to anticholinesterase activity and that PBO synergism enhanced toxicity to insects. Selectivity of these compounds was due to differential metabolic pathways, which may occur in insects and mammals. Toxicity of arylsulfenylated compounds to mice was lowered up to 50-fold in these compounds, but was 5-17 fold in alkyl substituents. With such confirmed improvement of mammalian safety, as well as increased toxicity to insects, a new generation of sulfenylated compounds was synthesized for testing. The compounds were derivatives of PRC 331 (G3 LC_{50} = 31 µg/mL) and 387 (G3 LC_{50} = 44 µg/mL), meant to improve the toxicity of parental compounds.

Sulfenylated analogs of PRC 331 and 387 did not show contact toxicity to mosquitoes, but PRC 655 was twice as toxic to G3 mosquitoes (LD_{50} = 2 ng), compared to PRC 331, the non-sulfenylated parental compound. The topical toxicity of the other compounds has not been determined yet, nor have I tested the toxicity of this series of compounds on Akron. It is therefore apparent that data obtained so far is preliminary, yet showing an interesting SAR. The lack of contact toxicity of PRC 655, even when it is highly toxic when topically applied cannot be fully addressed at this point, but possible future strategies are discussed in Chapter 6.

The phthalimide moiety, a known AChE peripheral site ligand (Yamamoto *et al.* 1994), was used to construct bivalent compounds, of which the pyrazoles PRC 590, PRC 591 and PRC 624 showed some low contact toxicity to G3 mosquitoes at 1 mg/mL. It remains to be determined if the *N, N*-dimethyl (PRC 591) and allophanate (PRC 624) analogs were metabolically bioactivated. PRC 590 showed improved toxicity to both susceptible and carbamate resistant mosquitoes when topically applied. All bivalent analogs of 590 with longer tether lengths were inactive, suggesting that three carbon or shorter tethers are optimal. When *in vitro* enzyme inhibition data and topical toxicity to both G3 and Akron mosquitoes become available, it will be possible to model the toxicity and anticholinesterase activity of related bivalent compounds. PRC 590 is therefore the first bivalent carbamate discovered by our group that is potent and also toxic to mosquitoes. This compound can be used as a platform to design and test related compounds, in the search for better mosquito toxicity and selectivity.

References

Akogbeto M, Djouaka R and Noukpo H (2005). Use of agricultural insecticides in Benin. *Bull. Soc. Pathol. Exot.* 98: 400-405.

Armstrong SJRM, Abdulla S, Nathan R, Mukasa O, Marchant TJ, Kikumbih N, Mushia AK, Mponda H, Minja H, Mshinda H, Tanner M and Lengeler C (2001). Effect of large-scale social marketing of insecticide-treated nets on child survival in rural Tanzania. *Lancet* 357: 1241-7.

Black AL, Chiu YC, Fahmy MAH and Fukuto TR (1973). Selective toxicity of N-sulfenylated derivatives of insecticidal methylcarbamate esters. *J. Agric. Food Chem.* 21(5): 747-751.

Carlier PR, Anderson TD, Wong DM, Hsu DC, Hartsel J, Ma M, Wong EA, Choudhury R, Lam PCH, Totrov MM and Bloomquist JR (2008). Towards a species-selective acetylcholinesterase inhibitor to control the mosquito vector of malaria, *Anopheles gambiae. Chemico-Biol. Inter.* 175: 368-375.

Carlier PR, Du DM, Han YF, Liu J, Perola E, Williams ID and Pang YP (2000). Dimerization of an inactive fragment of huperzine A produces a drug with twice the potency of natural product. *Angew. Chem. Int. Ed. Engl.* 39: 1775-1777.

Casida JE (1970). Mixed function oxidases involvement in the biochemistry of insecticides synergism. *J. Agric. Food. Chem.* 18: 753-772.

Casida JE, Gray RA and Tilles H (1974). Thiocarbamate sulfoxides: potent, selective and biodegradable herbicides. *Science* 184: 573-8.

Chen H, Githeko AK, Githure JI, Mutunga J, Zhou G and Yan G (2008). Monooxygenase levels and knockdown resistance (kdr) allele frequencies in *Anopheles gambiae* and *Anopheles arabiensis* in Kenya. *J. Med. Entomol.* 45(2): 242-250.

Coetzee M. and Hunt R.H. (1993). African Anopheline mosquito taxonomy and the control of malaria. *Entomologist extraordinary*: A festschrift in honor of Botha deMeillon. *Ed.* M. coetzee. S.A.I.M.R pp10.

Curtis CF, Miller JE, Hodjati MH, Kolaczinski JH and Kasumba I (1998). Can anything be done to maintain the effectiveness of pyrethroid-impregnated bednets against malaria vectors? *Philos. Trans. R. Soc. Lond.* B *Biol Sci.* 353(1376): 1769-1775.

Diallo DA, Cousens SN, Cuzin-Ouattara N, Nebié I, Ilboudo-Sanogo E and Esposito F (2004). Child mortality in a West African population protected with insecticide-treated curtains for a period of up to 6 years. *Bull. of the World Health Org.* 82: 85-91.

Fanello C, Kolaczinski JH, Conway DJ, Carnevale P, Curtis CF (1999). The kdr pyrethroid resistance gene in *Anopheles gambiae*: tests of non-pyrethroid insecticides and a new detection method for the gene. *Parassitologia.* 41(1-3): 323-326.

Feng S, Wang ZF, He XC, Zheng SX, Xia Y, Jiang HL, Tang XC and Bai DL (2005). Bis-huperzine B: highly potent and selective acetylcholinesterase inhibitors. *J. Med. Chem.* 48: 655-657.

Fukuto TR, Fahmy MAH and Metcalf RL (1967). Alkaline hydrolysis, anlichloin-esterase, and insecticidal properties of some nitro-substituted phenyl carbamates. *J. Agri. Food Chem.* 15: 273-281.

Geissbühler Y, Chaki P, Emidi B, Govella NJ, Shirima R, Mayagaya V, Mtasiwa D, Mshinda H, Fillinger U, Lindsay SW, Kannady K, Caldas de Castro M, Tanner M and Killeen GF (2007). Interdependence of domestic malaria prevention measures and mosquito-human interactions in urban Dar es Salaam, Tanzania. *Malar J.* 6:126.

Georghiou, GP. (1990a). The effect of agrochemicals on vector populations. In: Pesticide resistance in arthropods, *eds*. R. T. Roush; and B. E. Tabashnik. New York: Chapman and Hall. pp 183-202.

Guillet P, N'Guessan R, Darriet F, Traore-Lamizana M, Chandre F and Carnevale P (2000). Combined pyrethroid and carbamate 'two-in-one' treated mosquito nets: field efficacy against pyrethroid-resistant *Anopheles gambiae* and *Culex quinquefasciatus*. *Med. Vet. Entomol*. 15(1): 105-112.

Hadaway AB (1971). Some factors affecting the distribution and rate of action of insecticides. *Bull. World Health Org*. 44(1-2-3): 221-224.

Hadaway AB and Barlow F (1965). The relative toxicity of adult mosquitos of derivatives of phenyl N-methylcarbamates. *Bull. World Health Org*. 32(4): 581-585.

Harel M, Schalk I, Ehret-Sabatier L, Bouet F, Goeldner M, Hirth C, Axelsen P, Silman I, Sussman J (1993). Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. *Proc Natl Acad Sci U S A* 90: 9031-9035.

Hawley WA, Phillips-Howard PA, Terkuile FO, Terlouw DJ, Kolczak MS and Hightower AW (2003). Community-wide effects of permethrin-treated bed nets on child mortality and malaria morbidity in western Kenya. *Am. J. Trop. Med. Hyg*. 68(4): 121-127.

He XC, Feng S, Wang ZF, Shi YF, Zheng SX, Xia Y, Jiang HL, Tang XC and Bai DL (2007). Study on dual-site inhibitors of acetylcholinesterase: highly potent derivatives of bis- and bifunctional huperzine B. *Bioorg. Med. Chem*. 15: 1394-1408.

Hemingway J, Hawkes NJ, McCarroll L, Ranson H (2004). The molecular basis of insecticide resistance in mosquitoes. *Insect Biochem. Mol. Biol*. 34: 653–665.

Howard PA, Nahlen BL, Kolczak MS, Hightower AW, ter Kuile FO, Alaii JA, Gimnig Je, Arudo J, Vulule JM, Odhacha A, Kachur SP, Schoute E, Rosen DH, Sexton JD, Oloo AJ and Hawley WA (2003). Efficacy of permethrin-treated bed nets in the prevention of mortality in young children in an area of high perennial malaria transmission in western Kenya. *Am J Trop Med Hyg* 68:23-9.

Howard SC, Omumbo J, Nevill CG, Some ES, Donnelly CA, Snow RW (2000). Evidence for a mass community effect of insecticide treated bednets on the incidence of malaria on the Kenyan coast. *Trans. R. Soc. Trop. Med. Hyg*. 94: 357-360.

Karunaratne SHPP, Hawkes NJ, Perera MDB, Ranson H and Hemingway J (2007). Mutated sodium channel genes and elevated monooxygenases are found in pyrethroid resistant populations of Sri Lankan malaria vectors. *Pest. Biochem. Physiol*. 88(1): 108-113.

Khot AC, Bingham G, Field LM, and Moores GD (2008). A novel assay reveals the blockade of esterases by piperonyl butoxide. *Pest Manag Sci* 64:1139-1142.

Killeen GF, Smith TA, Ferguson HM, Mshinda H, Abdulla S, Lengeler C and Kachur SP (2007). Preventing childhood malaria in Africa by protecting adults from mosquitoes with insecticide-treated nets. *PLoS Med*. 4:e229.

Kolaczinski JH, Fanello C, Hervé JP, Conway DJ, Carnevale P and Curtis CF (2000). Experimental and molecular genetic analysis of the impact of pyrethroid and non-pyrethroid insecticide impregnated bednets for mosquito control in an area of pyrethroid resistance. *Bull. Entomol. Res*. 90(2):125-32.

Lengeler C (2004). Insecticide-treated bednets and curtains for preventing malaria. *Coch. Datab. of Syst Rev*. Oxford, United Kingdom: The Cochrane Library.

Lengeler C, Cattani J and Savigny D. eds (1996). Net gain. A new method for preventing malaria deaths. *IDRC*, Ottawa and WHO Geneva.

Lines JD (1988). Do agricultural insecticides select for insecticide resistance in mosquitoes? A look at the evidence. *Parasitology Today* 4: 17-20.

Ma HJ, Xie RL, Zhao QF, Mei XD and Ning J (2010). Synthesis and insecticidal activity of novel carbamate derivatives as potential dual-binding site acetylcholinesterase inhibitors. *J. Agric. Food Chem*. 58: 12817-12821.

Metcalf RL (1971). Structure-activity relationships of carbamates. *Bull. World Health Org*. 44(1-2-3): 43-78.

Metcalf RL and Fukuto TR (1965). Silicon-containing carbamate insecticides. *J. Econ. Entomol*. 58(6): 1151-9.

Miller JE, Lindsay SW and Armstrong JRM (1991). Experimental hut trials of bednets impregnated with synthetic pyrethroid or organophosphate insecticide for mosquito control in The Gambia. *Med. Vet. Entomol*. 5(4):465–476.

N'guessan R, Darriet F, Guillet P, Carnevale P, Traore-Lamizana M, Corbel V, Koffi AA and Chandre F (2003). Resistance to carbosulfan in *Anopheles gambiae* s.s. from Ivory Coast based on reduced sensitivity of acetylcholinesterase. *Med. Vet. Entomol*. 17: 1-7.

Ohkawa H and Casida JE (1971). Glutathione S-transferases liberate hydrogen cyanide from organic thiocyanates. *Biochem. Pharmacol*. 20: 1708-1714.

Overgaard HJ, Sandve SR and Suwonkerd W (2005). Evidence of anopheline mosquito resistance to agrochemicals in northern Thailand. *Southeast Asian J. of Trop. Med. and Pub. Health* 36(4): 148-153.

Overgaard HJ (2006). Malaria mosquito resistance to agricultural insecticides: Risk area mapping in Thailand. Colombo, Sri Lanka: International Water Management Institute. (*IWMI Research Report* 103). pp 1-68.

Pang YP, Quiram P, Jelacic T, Hong F and Brimijoin S (1996). Highly potent, selective, and low cost bis-tetrahydroaminacrine inhibitors of acetylcholinesterase: steps toward novel drugs for treating Alzheimer's disease. *J. Biol. Chem*. 271: 23646-9.

Pang Y-P, Singh SK, Gao Y, Lassiter TL, Mishra RK, Zhu KY and Brimijoin S (2009). Selective and irreversible inhibitors of aphid acetylcholinesterases: Steps toward human-safe insecticides. *PLoS ONE*. 2009; 4(2): e4349.

Piazzi L, Rampa A, Bisi A, Gobbi S, Belluti F, Cavalli A, Bartolini M, Andrisano V, Valenti P, Recanatini M (2003). 3-(4- [[Benzyl(methyl)amino]methyl]phenyl)-6,7-dimethoxy-2H-2-chromenone (AP2238) inhibits both acetylcholinesterase and acetylcholinesterase-induced beta-amyloid aggregation: a dual function lead for Alzheimer's disease therapy. *J. Med. Chem*. 46: 2279-2282.

Pluess B, Tanser FC, Lengeler C and Sharp BL (2010). "Indoor residual spraying for preventing malaria". *Cochrane Database Syst. Rev*. 4: CD006657.

Pridgeon JW, Pereira RM, Becnel JJ, Allan SA, Clark GG and Linthicum KJ (2008). Susceptibility of *Aedes aegypti*, *Culex quinquefasciatus* Say, and *Anopheles quadrimaculatus* Say to 19 pesticides with different modes of action. *J. Med. Entomol*. 45: 82–87.

Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z and Corbel V (2010). Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trend. in Parasitol*. 27(2): 91-98.

Risher JF, Mink FL, and Stara JF (1987). The toxicological effects of the carbamate aldicarb in mammals. A review. *Environ Health Perspect*. 72: 267-281.

Rowland M and N'Guessan R (2009). Pyrethroid resistance in the African mosquito *Anopheles gambiae* and alternative insecticides for indoor residual spraying and use on mosquito nets (In) Advances in Human Vector Control. *ACS Symp. Ser*. Vol. 1014 (2): 13-25.

Sanchez-Arroyo H, Koehler PG and Valles SM (2001). Effects of the synergists piperonyl butoxide and S,S,S-Tributyl phosphorotrithioate on propoxur pharmacokinetics in *Blattella germanica* (Blattodea: Blattellidae). *J. Econ. Entomol*. 94(5): 1209-1216.

Scott JA, Brogdon, WG and Collins FH (1993). Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *Am. J. Trop. Med. Hyg*. 49: 520-529.

Steketee RW, Sipilanyambe N, Chimumbwa J, Banda JJ, Mohamed A, Miller J, Basu S, Miti SK and Campbell CC (2008). National malaria control and scaling up for impact: The Zambia experience through 2006. *Am. J. Trop. Med. Hyg*. 79(1): 45-52.

Sun YP and Johnson ER (1972). Quasi-synergism and penetration of insecticides. *J. Econ. Entomol.* 65: 349 -353.

Sussman J, Harrel M, Frolow F, Oefner C, Goldman A, Toker L and Silman I (1991). Atomic structure of acetylcholinesterase from *Torpedo carlifornica*: a prototypic acetylcholine binding protein. *Science* 253:872-879.

ter Kuile FO, Terlouw DJ, Phillips-Howard PA, Hawley WA, Friedman JF, Kariuki SK, Shi YP, Kolczak MS, Lal AA, Vulule JM and Nahlen BL (2003). Reduction of malaria during pregnancy by permethrin-treated bed nets in an area of intense perennial malaria transmission in western Kenya. *Am. J. Trop. Med. Hyg.* 68: 50-60.

Weill M, Malcolm C, Chandre F, Mogenson K, Berthomieu A, Marquine M and Raymond M. (2004). The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. *Insect Mol. Biol*. 13: 1-7.

W.H.O (1981). Document *WHO/VBC/81.805*, World Health Organization, Geneva.

W.H.O. (1989). The use of Insecticide impregnated bednets and other materials for vector borne disease control. In *WHO/VBC/89.981*. WHO Geneva.

W.H.O. (2000). The Abuja Declaration and the Plan of Action, The African Summit on Roll Back Malaria, Abuja, 25 April 2000 (*WHO/CDS/RBM/*2000.17).

W.H.O. (2008). World Malaria Report 2008, World Health Organization.

W.H.O. (2010). Malaria vector control bulletin. http://www.who.int/malaria/vector_control/en/.

Wong DM, Greenblatt HM, Dvir H, Carlier PR, Han Y, Pang YP, Silman I and Sussman J (2003). Acetylcholinesterase complexed with bivalent ligands related to Huperzine A: Experimental evidence for species-dependent protein-ligand complementarity *J. Am. Chem. Soc.* 125(2): 363-373.

Yamamoto Y, Ishihara Y, and Kuntz ID (1994). Docking analysis of a series of benzylamino acetylcholinesterase inhibitors with a phthalimide, benzoyl, or indanone moiety. *J. Med. Chem.* 37 (19): 3141–3153.

Zaim M, Aitio A and Nakashima N (2000). Safety of pyrethroid-treated mosquito nets. *Med. Vet. Entomol.* 14(1): 1-5.

Zhao QF, Yang GQ, Mei XD and Ning J (2008). Design of novel carbamate acetylcholinesterase inhibitors based on the multiple binding sites of acetylcholinesterase. *J. Pestic. Sci.* 33: 371-375.

Zhao QF, Yang GQ, Mei XD and Ning J (2009). Novel acetylcholinesterase inhibitors: synthesis and structure-activity relationships of phthalimide alkyloxylphenylN,N-dimethylcarbamate derivatives. *Pestic. Biochem. Physiol.* 95: 131-134.

Chapter 4. The Structure-Activity Relationships of Halogenated, Thioalkyl and Side-Chain Branched Phenyl *N*-Methylcarbamates

Abstract

In this chapter, I describe the enzyme selectivity (*Anopheles gambiae* vs. human) and toxicity of modified structural analogs of phenyl-substituted carbamates. Structural modifications include halogenation and thiolation of the phenyl ring and the alkyl/silyl side-chains, and extended side-chain branching. Halogenation did not enhance anticholinesterase potency, but some fluorinated compounds exhibited significant mosquito toxicity. Side-chain branching and thiolation structure-activity relationships (SAR) both revealed the need for molecular and structural complementarity to the AChE and ACh. In addition, carbamoylated phenyl substituents and trifluoromethylketone derivatives were inactive, while silicon-containing analogs of active molecules retained activity. Overall, none of the proposed modifications led to both enhanced mosquito selectivity and toxicity, compared to the parental carbamates. Greater enzyme selectivity was observed in some cases, but this would often lead to loss of toxicity, *in vivo*. Conversely, some analogs that were poor anticholinesterases were appreciably toxic to mosquitoes, thereby indicating that both the affinity to AChE and the metabolic fate of carbamates are important considerations in the design of mosquito-selective inhibitors as candidate carbamate insecticides. Finally, a series of substituted benzylpiperidines designed as bivalent inhibitors spanning both the catalytic and peripheral sites, were also studied. All were inactive, except one compound that was selective at the enzyme level, but possessed no *in vivo* toxicity. The implications of these results for design of new mosquitocides is discussed.

4.1 Introduction

The use of commercial carbamate insecticides dates back to the late 1950s (Gysin and Margot, 1958), and since then numerous efforts have been made to improve their toxicity to insects. Despite the wide-scale use of carbamates for crop and urban pest control (Plimmer, 2001), only propoxur and bendiocarb are authorized by the World Health Organization (WHO) for indoor residual spraying of mosquitoes (WHO, 2002a). Current advances in proteomics and genomics, the availability of both the *Anopheles gambiae* and human genomes, and advanced computational biology tools enable fine-scale ligand docking, virtual ligand library screening and synthesis of new highly potent AChE inhibitors. An example is the *bis*-Huperzine B inhibitors that are highly potent and selective for AChE and are candidates for the treatment of Alzheimer's disease (Feng *et al*. 2005).

Despite great advances in discovery of selective and potent inhibitors of HuAChE, much remains to be achieved in developing mosquito-selective carbamates. Most of the early pioneer experiments on carbamate toxicity and selectivity were based on housefly and mouse toxicity data. For example, LD_{50} rat/LD_{50} housefly ratios range from > 100 for butacarb to 0.2 for aldicarb (Metcalf, 1971), indicating that a wide range of selectivity can be achieved depending on the insect species and on the chemical tested. Two primary factors determine the selectivity of a carbamate: selective detoxification in vertebrate systems and selective AChE inhibition to target insect species (Metcalf *et al*. 1971).

This chapter describes effects of various structural modifications made to PRC 331 and PRC 387 to mosquitoes. The modifications include extended side-chain branching of alkyl or silyl substituent and halogen stereochemistry. Kolbezen *et al.* (1954) suggested that structural complementarity of the *tert*-butyl group to ACh was attributable to high potency with bovine

AChE. Accordingly, side-chain branched carbamates explored effects of spatial and structural complementarity of compounds to Me_3N^+ of ACh. Halogenation explored not only the size of halogen substituents, but also their preferred placement on the molecule. Since *N*-demethylation is an unlikely detoxication mechanism of *N*-methylcarbamates, *in vivo* (Fahmy, 1966), carbamate side-chains are conventionally the major detoxication sites. Halogenation at these sites is deemed to confer protection against hydroxylation by P450 monooxygenases because of the strong carbon-halogen bonds (Park *et al.* 2001). Inclusion of thioalkyl substituents sought to explore not only the ACh structural complementarity of these analogs, but also the selective bioactivation of sulfur to more toxic sulfoxide and sulfone derivatives, *in vivo*. This SAR data helps to understand interactions of carbamates with AChE, and informs the future design of a pharmacophore with the inherent properties of superior toxicity and selectivity.

Efforts to make a bivalent anticholinesterase are also reported. The use of a bivalent insecticide which carries a catalytic active site inhibitor specific for the mosquitoes, and alkyl-linked to another ligand whose specific peripheral site binding enhances the potency and toxicity of the overall compound, is one strategy to mitigate resistance development in insects. Such bivalent inhibitors will also likely be effective against carbamate-resistant mosquitoes having the MACE form of target site resistance.

4.2 Materials and methods

4.2.1 *Chemicals and reagents*

All experimental compounds were synthesized and purified to >95% by the Carlier group at the Department of Chemistry, Virginia Tech. Acetylthiocholine, HuAChE, DTNB and all

buffer components were purchased from Sigma-Aldrich (MO, USA). Whole mosquito homogenate was used as a source of *Ag*AChE.

4.2.2 *AChE inhibition assays*

AChE enzyme inhibition assays were performed using the Ellman method (1969), adapted for a 96-well microplate assay as described in Chapter 2, with a few modifications. Briefly, inhibitor stocks of 0.01 M were freshly prepared in DMSO followed by serial dilutions in DMSO. A 100-fold dilution into sodium phosphate buffer (pH 7.8) was made for each of the DMSO dilutions. The final DMSO concentration was maintained at 0.1% v/v. After a 10 min pre-incubation of the enzyme and inhibitor, hydrolysis of the Ellman's reagent (ATChI and DTNB) was monitored for 10 mins at 405 nm, in a Dynex 96-well plate reader. Percent residual AChE activity values (relative to the control) were plotted in Prism® (Graphpad, USA) and analyzed by non-linear regression (curve-fit) to generate IC_{50} values. IC_{50} is the inhibitor concentration that inhibits 50% of the enzyme, and defines the potency of the inhibitor. Mosquito selectivity (S) was determined by the IC_{50} ratio of HuAChE and AgAChE for each compound.

$$\text{Selectivity } (S) = \frac{Ag\text{AchE IC}_{50}}{\text{HuAchE IC}_{50}}$$

Significance of both potency and toxicity of the compounds was assessed based on non-overlapping of 95% confidence intervals (95% CI).

4.2.3 Insect bioassays

A standard WHO treated filter paper assay (WHO, 1981) was used to assess contact toxicity of the structural analogs, relative to previously described toxicity of parental carbamates (in chapter 3). An initial range finding assay was performed with 3 concentrations of 0.1, 0.5 and 1 mg/mL for each compound in 95% ethanol, to assess the particular range within which more test concentrations would be made to obtain an LC_{50} value. From this initial assay, only compounds showing greater than 50% mortality at 0.5 mg/mL were further tested using a narrow range of concentrations, with up to 5 concentrations per compound and 25 mosquitoes per treatment. I used 2-5 day old sugar-fed females. Assays were repeated at least twice using different batches of mosquitoes to account for inter-batch variability. Treated mosquitoes and untreated controls were maintained at 75% RH and 78° F. Mortality was assessed after 24 hrs and corrected for control mortality, if any as described in chapter 3. Data was plotted in Poloplus® and a probit analysis performed to generate LC_{50} values; the carbamate concentration that kill 50% of the exposed mosquitoes.

4.3 Results

4.3.1 Effects of additional alkyl groups on the tert-*butyl phenyl substituent of PCR331 to AChEs and mosquito toxicity*

Addition of alkyl groups that extend the *tert*-butyl moiety of PRC 331 (Fig. 4.1), resulted in variation of potency to both AChEs, as well as changes in mosquito toxicity (Table 4.1).
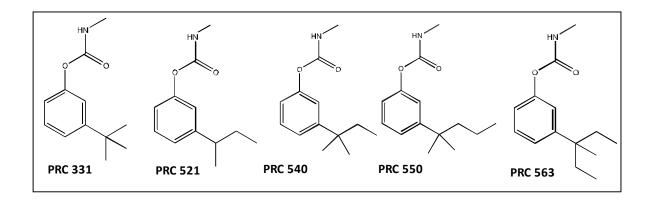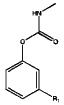
Figure 4. 1. Chemical structures of PRC 331 and its alky elongated analogs.

Table 4. 1. AChE inhibition and mosquito toxicity of PRC 331 and its *tert*-butyl group elaborated analogs.

General structure of PRC 331
analogs listed in table 4.1

Cmpd	R_1	AgAChE IC_{50}[*], nM	HuAChE IC_{50}[*], nM	S	LC_{50}[*], μg/mL
PRC 331	*tert*-butyl	36 (34-38)[a,1]	320 (293-349)[a,2]	9	31 (14-60)[a]
PRC 521	*sec*-butyl	17 (15-19)[b,1]	25 (20-31)[b,1]	1.5	31 (29-33)[a]
PRC 540	*tert*-pentyl	35 (33-36)[a,1]	48 (37-60)[c,2]	1.4	68 (64-72)[b]
PRC 550	2-Me-pentan-2-yl	11 (7-18)[b,1]	12 (6-24)[b,1]	1.1	236 (210-259)[c]
PRC 563	3-Me- pentan-3-yl	47 (33-66)[a,1]	42 (22-82)[c,1]	0.9	228 (214-228)[c]

[*]Values in brackets are 95% CI. S= Mosquito selectivity = HuAChE IC_{50}/AgAChE IC_{50}. IC_{50}s in the same column and row followed by the same superscript letter and number, respectively, are not statistically different ($P > 0.05$) based upon non-overlap of the 95% CI.

Substitution of the *tert*-butyl with a *sec*-butyl (PRC 331 to PRC 521) led to a ca. 2-fold gain in *Ag*AChE potency, and a 13-fold gain with HuAChE, concurrently abolishing the selectivity of PRC 331, while there is no change in mosquito toxicity (Table 4.1). With the *tert*-

pentyl group (PRC 540), there was ca. 7-fold gain with HuAChE, when compared to PRC 331. This change also resulted to a 2-fold loss in mosquito toxicity. Extending the PRC 540 chain further by a methyl group to PRC 550 leads to an AChE potency pattern similar to the *sec*-butyl substituent (PRC 521). Despite the ca. 3-fold gain in potency to *Ag*AChE, there was nearly 8-fold loss in mosquito toxicity, when compared to the parent compound (PRC 331). There was *ca*. 4-fold loss in both Ag and HuAChE potency (Table 4.1), but no significant change in toxicity, observed when a methyl group was added to PRC 550 (to make PRC 563).

4.3.2 Structure activity relationships of thioalkyl N-*methylcarbamate analogs of PRC 331*

The structure of PRC 331 was redesigned to contain a thio-alkyl substituent attached to the phenyl ring (Fig 4.2). The effects on both AChE potency and mosquito toxicity are summarized in Table 4.2.
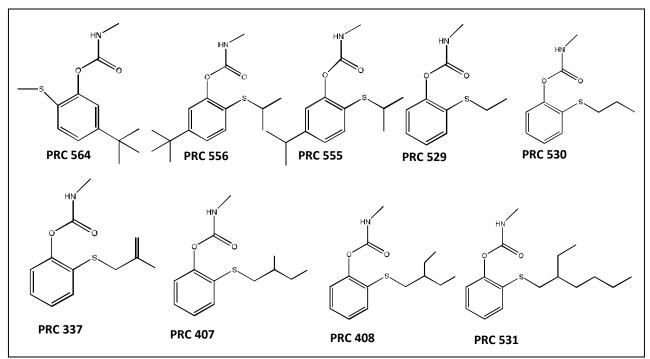
Figure 4. 2. Chemical structures of PRC 331 and its analogs bearing phenyl substituted thioalkyl groups.

Oxidation of thioalkyl groups in the mosquito body may generate the more toxic sulfoxide or sulfone derivatives (Fig. 4.3), and therefore this series of compounds was intended to be bioactivated by the mosquito metabolic system.

Figure 4. 3. Metabolic bioactivation pathway of thioalkyl groups to more toxic sulfoxide and sulfone derivatives, *in vivo*.

Addition of a Me-*S*- group at a position *para* to the *tert*-butyl group (PRC 564) caused a drastic 27-fold loss in potency to *Ag*AChE, compared to PRC 331, the reference compound (Table 4.2). This also caused a complete loss of toxicity to mosquitoes at 1000 µg/mL, our diagnostic maximum dose. Interestingly, this modification led to a mere 2-fold loss in potency to *Hu*AChE. Extending the thio-methyl by 2 carbons to make an isopropylthio substituent (PRC 556) leads to 1061-fold and 40-fold loss in potency to *Ag*AChE, when compared to PRC 564 (methyl-thio) and PRC 331, respectively. In HuAChE, the same modification led to a 7-fold and 15-fold loss of potency, when compared to PRC 564 and PRC 331, respectively (Table 4.2). PRC 556 was marginally toxic to mosquitoes. In line with this SAR, we tested an analog of PRC 556, whose *tert*-butyl group was changed to *iso*-propyl, but still retained the *para*-isopropylthio moiety (PRC 555). This modification was meant to investigate whether thiolation of the phenyl group would improve toxicity, when the *tert*-butyl moiety is altered. Compared to PRC 556, the removal of a methyl from *tert*-butyl (PRC 555) led to a 2-fold loss and 2-fold gain in potency to *Ag*AChE, and *Hu*AChE, respectively (Table 4.2).

Table 4. 2. Potency and toxicity of thioalkyl N-methylcarbamates to *An. gambiae* mosquitoes

General structure of thioalkyl *N*-methylcarbamates listed in table 4.3.2

Compound	R_1	R_2	AgAChE-IC$_{50}$*, nM	HuAChE-IC$_{50}$*, nM	S	LC$_{50}$*; µg/mL or % mortality
PRC 564	*t*-butyl	Me-S	974 (830-1172)[a,1]	641 (572-719)[a,2]	0.66	0% @ 1mg/mL
PRC 556	*t*-butyl	*i*-Propyl-S	32210 (23060-63300)[b,1]	4674 (3777-5784)[b,1]	0.12	4% @ 1mg/mL
PRC 555	*i*-propyl	*i*-Propyl-S	75440 (N/A)	2205 (1653-2941)[c]	0.03	16% @ 1mg/mL
PRC 529	H	Et-S	243 (216-276)[c]	> 100,000 (N/A)	>412	296 (279-314)[a]
PRC 530	H	Propyl-S	114 (135-152)[d,1]	2290 (2019-2596)[c,2]	16	339 (321-361)[b]
PRC 337	H	2-Me-allyl-S	77 (59-102)[e,1]	5778 (5439-6123)[b,2]	75	69 (53-58)[c]
PRC 407	H	2-Me-butyl-S	36 (27-49)[f,1]	1219 (1092-1361)[d,2]	34	70% @ 1mg/mL
PRC 408	H	2-Et-butyl-S	37 (32-43)[f,1]	5036 (4514-5618)[b,2]	136	27% @ 1mg/mL
PRC 531	H	2-Et-hexyl-S	40760 (30940-53680)[b,1]	3527 (2261-5503)[b,1]	0.09	0% @ 1mg/mL

*Values in brackets are 95% CI. S= mosquito selectivity as defined in Eqn 4.1. IC$_{50}$s in the same column or row followed by the same superscript letter and number, respectively, are not statistically different (P > 0.05).

Toxicity of PRC 555 to mosquitoes remained low (16% at 1000 μg/mL). The removal of the *tert*-butyl group and shortening the thioalkyl substituent of PRC 555 by a methyl made the ethylthio analog (PRC 529). These modifications led to improved *Ag*AChE potency (310-fold), compared to PRC 555, and completely rendered the compound inactive to HuAChE at 100,000 nM. Consequently, PRC 529 had an estimated *Ag*AChE selectivity of > 412, the highest value observed so far in this series of analogs. More so, PRC 529 was toxic to mosquitoes, with an LC_{50} of 296 μg/mL. Addition of an extra methyl group to the ethylthio substituent of PRC 529 to make a propylthio group (PRC 530) increased potency to *Ag*AChE by ca. 2-fold, although no change in mosquito toxicity was observed. PRC 530 also showed significant activity towards HuAChE, unlike PRC 529.

Substituting the propylthio group of PRC 530 with a thioallyl group (PRC 337) led to nearly 2-fold potency gain with *Ag*AChE and *ca*. 3-fold loss with HuAChE, consequently increasing mosquito selectivity from 16 to 75 (Table 4.2). The change to thioallyl also led to a 5-fold increase in mosquito toxicity, not entirely consistent with the slight increase in *Ag*AChE inhibitory potency. Further elongation of the thioalkyl chain by a methyl group (PRC 407) yielded a 2-fold gain in *Ag*AChE potency, whereas inhibition of HuAChE remained unchanged. This modification also led to loss of mosquito toxicity (70% mortality at 1 mg/mL) compared to PRC 337 (Table 4.2). Addition of another methyl group onto the 2-methylbutylthio to make a 2-ethylbutylthio (PRC 408) left *Ag*AChE IC_{50} unchanged, whereas potency to HuAChE dropped 4-fold, thereby leading to an increased mosquito selectivity of 136, the second highest in this series of compounds (Table 4.2). Although there was no loss in *Ag*AChE potency with PRC 408, we observed a decrease in mosquito toxicity compared to PRC 407, its closely related analog. Addition of a methyl group onto PRC 408, (to make 2-ethylhexylthio substituent; PRC 531),

106

caused a > 1100-fold loss in *Ag*AChE potency. In contrast, there was a slight but insignificant gain in potency to HuAChE. Consequently, both the superior selectivity observed with the 2-ethylbutyl analog (PRC 408) and the mild toxicity to mosquitoes were lost (Table 4.2).

4.3.3 AChE potency and mosquito toxicity of carbamoyl analogs of tert-*butyl* N-*methylcarbamate*

The activity of additional substitutions to the phenyl ring was evaluated, while maintaining the *tert*-butyl group of PRC 331 unchanged (Fig. 4.4). These modifications included an alkyl linker at C_2, *para* to the *tert*-butyl group as a strategy to tether the PRC 331 to a peripheral active site specific ligand, and carbamoylating the phenyl ring at a position *ortho* to the *tert*-butyl group.

Figure 4. 4. Chemical structures of and carbamoyl analogs of PRC 331 used in the study.

The carbamoyl analogs of PRC 331 did not have either *Ag*AChE inhibitor potency or mosquito toxicity. In HuAChE however, we observed micromolar IC_{50}s with the allyl, *di*-ethyl and *N*-ethyl, *N*-methyl carbamoyl analogs, but no activity was observed with the methyl and *iso*-butyl analogs. Linkage of a hexyl group, *para* to the *tert*-butyl group, resulted in a 3000-fold

potency loss against *Ag*AChE when compared to PRC 331. Contrastingly, this hexyl analog is a mild inhibitor of HuAChE, with a ca. 175-fold loss in potency, relative to PRC 331.

Table 4. 3. AChE inhibition and mosquito toxicity of PRC 331 carbamoyl analogs

General structure of the carbamoyl and allophanate *N*-methylcarbamates listed in Table 4.3 below

Cmpd	R_1	R_2	AgAChE-IC_{50}, nM	HuAChE-IC_{50}, nM	S	% mortality
PRC 507	H	Hex.	>100, 000	6293 (5126-7460)	<0.06	4% @ 1mg/mL
PRC 565	Allyl carbamoyl	H	>100,000	2835 (1719-4677)	<0.03	0% @ 1mg/mL
PRC 566	Me-carbamoyl	H	>100,000	>100,000	N/A	4% @ 1mg/mL
PRC 567	*i*-But-carbamoyl	H	>100,000	>100,000	N/A	0% @ 1mg/mL
PRC 568	*Di*-Et-carbamoyl	H	>100,000	2299 (1189-4448)	<0.023	0% @ 1mg/mL
PRC 569	*N*-Et, *N*-Me-carbamoyl	H	>100,000	5479 (2830-8128)	<0.06	0% @ 1mg/mL

4.3.4 Comparative AChE potency and mosquito toxicity of halogenated and silyl N-*methylcarbamates*

Halogenated *N*-methylcarbamates were tested to explore the effects of halogenation on enzyme inhibition and toxicity (Fig. 4.5). Silicon bearing analogs were also tested and their inhibition potency and toxicity compared to the carbon bearing parental structures (Fig. 4.5).

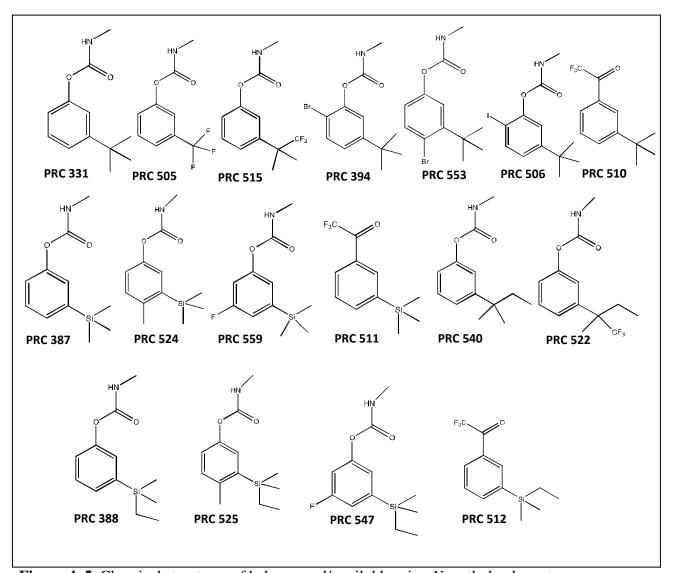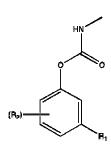**Figure 4. 5.** Chemical structures of halogen and/or silyl bearing *N*-methylcarbamates.

General structure of halogenated analogs presented in Table 4.4. The *meta*, *ortho* and *para* positions of the phenyl ring substituents (R_2) are relative to the R_1 group. IC_{50} values in the same column and row followed by the same superscript letter and number, respectively, are not statistically different ($P > 0.05$).

Table 4. 4. AChE inhibition and mosquito toxicity of halogenated and silyl *N*-methylcarbamate analogs of PRC 331

Compound	R_1	R_2	AgAChE IC_{50}*, nM	HuAChE IC_{50}*, nM	S	LC_{50}*[#] or % mortality
PRC 331	*tert*-butyl	H	36 (34-38)[a,g,1]	320 (293-347)[a,2]	9	31 (14-60)[a]
PRC 505	-CF₃	H	>10^{-5}M	>10^{-5}M	N/A	393 (333-471)[b]
PRC 515	*tert*-butyl-CF₃	H	345 (287-415)[b,1]	2426(1506-3346)[b,f,g,2]	7	61 (43-80)[a,f]
PRC 394	*tert*-But.	*para*-Br	14 (12-17)[c,1]	48 (40-56)[c,2]	3.4	257 (239-276)[c]
PRC 553	*tert*-But.	*ortho*-Br	576 (260-892)[b,i,1]	1223 (913-1533)[b,2]	2	599 (570-627)[d]
PRC 506	*tert*-But.	*para*-I	2146 (1195-2309)[d,1]	638 (538-752)[d,2]	0.3	20% @ 1 mg/mL
PRC 510	*tert*-But.	–CF₃ketone	7775 (6235-9317)[e,1]	37 (22-52)[c,2]	0.0048	8% @ 1 mg/mL
PRC 387	Me₃-Si-	H	50 (40-64)[f,1]	488 (452-526)[e,2]	9.8	169 (162-176)[e]
PRC 524	Me₃-Si-	*ortho*-Me	378 (318-450)[b,1]	2418 (2032-2814)[f,2]	6.4	241 (154-330)[e,c]
PRC 559	Met₃-Si-	*meta*-F	2622 (1260-5453)[d,1]	4161 (3714-4616)[g,1]	1.6	4% @ 1 mg/mL
PRC 511	Met₃-Si-	-CF₃ketone	30950 (29220-32680)[g,1]	852 (732-972)[d,2]	0.028	8% @ 1 mg/mL
PRC 540	*tert*-pentyl	H	35 (33-37)[a,1]	48 (37-60)[c,2]	1.4	68 (64-72)[f]
PRC 522	F₃C-butyl-2-yl	H	42 (38-46)[f,g,1]	104 (86-124)[h,2]	2.5	115 (95-147)[g]
PRC 388	Et-Me₂-Si	H	72 (67-78)[h,1]	626 (515-761)[d,e,2]	8.7	185 (154-229)[e]
PRC 525	Et-Me₂-Si	*ortho*-Me	630 (556-714)[i,1]	1379 (1205-1559)[b,2]	2.2	16% @ 1mg/mL
PRC 547	Et-Me₂- Si	*meta*-F	189 (158-225)[j,1]	1941 (1649-2245)[g,f,2]	10.3	439 (405-474)[b]
PRC 512	Et-Me₂-Si	-CF₃ketone	25200 (24550-25862)[k,1]	844 (691-998)[d,2]	0.033	0% @ 1mg/mL

*Values in brackets are 95% CI, [#]LC_{50} values in µg/mL. 'S' denotes mosquito selectivity (Eqn 4.1)

110

I tested the effect of bromination via substitutions *para* (PRC 394) and *ortho* (PRC 553) to the *tert*-butyl group. Bromine at the *para* position increases potency to both AChEs, and slightly reduces toxicity to mosquitoes (Table 4.4). Bromination at the *ortho* position leads to a 16-fold loss in *Ag*AChE potency, compared to a 4-fold loss in HuAChE, relative to PRC 331. Consequent to this loss of *Ag*AChE inhibition potency, toxicity to mosquitoes was also decreased 2-fold when compared to the para analog and 18-fold when compared to the unbrominated analog, PRC 331 (Table 4.4). Substitution of the *para* bromo with iodo (PRC 506) leads to over 150-fold and 13-fold loss in potency to *Ag*AChE and HuAChE, respectively. This implies that although halogenation at the *para* position is preferred over *ortho*, a smaller halogen is more favored.

The presence of fluorinated groups on or near the *tert*-butyl or trimethylsilyl bearing carbamates affected both AChE potency and mosquito toxicity (Table 4.4). When the *tert*-butyl group was substituted with trifluoromethyl (PRC 505), the potency to both mosquito and human AChEs was lost, but surprisingly the compound was toxic to mosquitoes. Flourination of a methyl at the *tert*-butyl group (PRC 515) caused 10-fold and 8-fold decrease in *Ag*AChE and HuAChE, respectively, when compared to PRC 331. Despite this potency loss, the compound is only half as toxic to mosquitoes. Fluorination at the *meta* position of the trimethylsilyl group (PRC 559) caused a 50- and a 9-fold loss of potency to *Ag*AChE and HuAChE, respectively and consequently rendered the compound non-toxic to mosquitoes (Table 4.4). Fluorination of PRC 540 to make PRC 522 does not improve either potency to AChEs or toxicity to mosquitoes. A fluorine *meta* to the ethyl-dimethylsilyl group of PRC 388 (PRC 547) reduces *Ag*AChE 9-fold, compared to a 2-fold loss in HuAChE. This decreased *Ag*AChE potency is associated with a ca. 2-fold loss in toxicity.

Trifluoromethylketone replacement of the methyl carbamate moiety was also attempted. Derivatives of the *tert*-butyl (PRC 510), trimethylsilyl (PRC 511) and the ethyl-dimethylsilyl (PRC 512) analogs are low micromolar inhibitors of *Ag*AChE that exhibit ca. 210, 30, and 36-fold higher potency to HuAChE, respectively, and no toxicity to mosquitoes, relative to their respective reference carbamates (Table 4.4). Additional methyl ring substitutions were attempted. A methyl group placed *ortho* to the trimethylsilyl substituent (PRC 524) of PRC 387 causes a significant potency loss in both mosquito and human AChEs, but the change in mosquito toxicity is insignificant. A methyl addition *ortho* to the ethyl-dimethylsilyl group of PRC 388 to make PRC 525 led to loss of both AChE inhibition potency and toxicity to mosquitoes (Table 4.4).

4.3.5 Enzyme inhibition and mosquito toxicity of piperidine-derived AChE bivalent ligands

Some bivalent structures containing phthalimide peripheral site ligands were described in Chapter 3, and here I report the potency and toxicity of piperidine-containing bivalent non-carbamate ligands (Fig. 4.5 and Table 4.5).
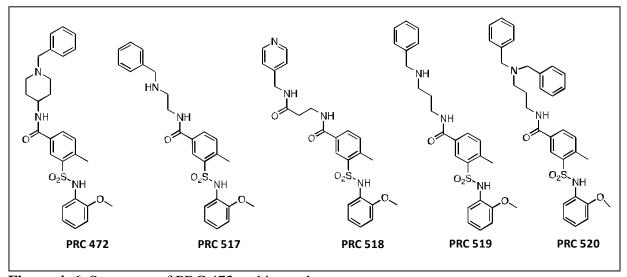
Figure 4. 6. Structures of PRC 472 and its analogs.

These studies were undertaken in light of the discovery of PRC 472 (Fig. 4.5), which was a nanomolar inhibitor of the mosquito enzyme (Table 4.5), and shows ca. 75-fold selectivity towards *Ag*AChE, the highest value so far observed with our bivalent ligands. Structural changes were made at the benzylpiperidine moiety of PRC 472, in order to generate analogs with varying two methylene (PRC 517) and three methylene (PRC 518) spacers, linked to variable structural substituents (PRC 517 to PRC 520). The aim was to evaluate not only the optimal spacing of this PSL, but also to explore the basicity of piperidine nitrogen atom. PRC 517 bears a basic amine whereas PRC 518 is a non-basic analog.

The substitution of the benzyl moiety in PRC 472 with a pyridinyl (PRC 518) caused a drastic loss in potency to both *Ag* and *Hu*AChE. While maintaining the benzyl R group of PRC 472, the substitution of the piperidine with an ethylamine (PRC 517) or a propylamine (PRC 519) caused reduced potency and selectivity. However, addition of an extra benzyl group on PRC 519 to create PRC 520 led to complete insensitivity to both enzymes, indicating that the extra benzyl group resulted in unfavorable ligand-enzyme interactions. Despite the markedly low *Ag*AChE IC_{50}, PRC 472, as well as its analogs, were not toxic to mosquitoes, neither by contact, topical application, nor injection bioassays.

Table 4. 5. AChE potency and mosquito toxicity of PRC 472, and its analogs.

General structure of AChE PSLs

shown in Fig. 4.5

Cpd No.	R	AgAChE IC$_{50}$,* nM (95% CI)	HuAChE IC$_{50}$,* nM (95% CI)	S	% 24hr mortality
PRC 472	Benzylpiperidine	166 (152-182)[a,1]	12470 (11800-13180)[a,2]	75	0% @ 1mg/mL
PRC 517	*N*-benzyl ethanamine	11820 (9034-15460)[b,1]	33940 (29930-38490)[b,2]	3	8% @ 1mg/mL
PRC 518	Pyridinyl-4-yl-Me-propionamide	>100,000	>100,000	N/A	0% @ 1mg/mL
PRC 519	*N*-benzyl propan-1-amine	2920 (2700-3157)[c,1]	36990 (18990-70200)[b,2]	13	0% @ 1mg/mL
PRC 520	*N,N*-dibenzyl propan-1-amine	>100,000	>100,000	N/A	0% @ 1mg/mL

*Values in brackets are 95% CI. 'S' denotes mosquito selectivity (Eqn 4.1). IC$_{50}$ values in the same column and row followed by the same superscript letter and number, respectively, are not statistically different ($P > 0.05$).

4.3.6   Correlation analysis of potency data with in vivo *toxicity data of* N-*methylcarbamates*

I plotted the IC_{50} and LC_{50}s of compounds that displayed a fair correlation between toxicity and potency (Zone A) to generate Figure 4.6. Using this correlation as a template, I was able to partition the probable positions on the plot, of compounds that showed either high enzyme potency with poor toxicity (zone B) or high toxicity with low enzyme potency (zone C). The AgIC$_{50}$s or LC_{50}s of zone B and C compounds were not plotted because some compounds did not have actual measurable values. Also, the scale of the plot is based on compounds in zone A, and therefore values for compounds in zone B and C may fall beyond this scale.   The implications of this analysis are discussed below.

Correlation of enzyme potency and toxicity in mosquitoes
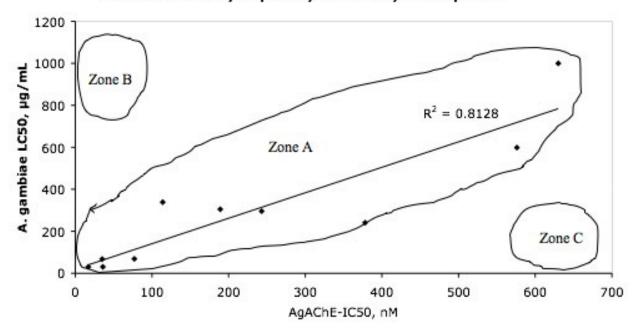
Figure 4. 7. Comparison of *Ag*AChE inhibition with mosquito toxicity for PRC compounds. Data plotted is for PRCs 331, 337, 521, 525, 529, 530, 540, 542, 547 and 553 (zone A). Examples of zone B include PRC 407, PRC 408 and PRC 472. Examples of Zone C include PRC 505, PRC 515 and PRC 555.

115

4.4 Discussion

The search for carbamate insecticides that have superior insect selectivity and efficacy has been a challenging task since the discovery of carbamates in the early 1940s. Despite having high toxicity to insects, these usually broad-spectrum insecticides are also toxic to vertebrates and non-target invertebrates. I have structured this Discussion in subsections, to feature the different modifications, and make it easier for readers to follow. I hereby detail the effects of various modifications to both the inhibition potency of the analogs to mosquito and human AChE, and the changes in toxicity to mosquitoes.

4.4.1 Correlation of potency data with in vivo toxicity data of N-methylcarbamates

Compounds that displayed either or both potent enzyme inhibition and toxicity belonged to three general groups based on Fig. 4.6. The boundary limits for zones A-C are not based any statistical model and therefore inclusion or exclusion of a compound is based on empirical *in vitro* and *in vivo* data. Compounds within zone A are AChE inhibitors whose toxicity correlates well with enzyme data, and therefore do not undergo major metabolic activation or degradation. Compounds in zone B are good AChE inhibitors, but are either unable to penetrate the cuticle, blood brain barrier or undergo high metabolic detoxication, *in vivo*. If cuticular/hemolymph brain barrier penetration and/or metabolic degradation are suspect processes, then PBO-synergized LC_{50}s and injected-dose 50s (ID_{50}) of these compounds should fall under zone A. Compounds in zone C are poor AChE inhibitors *in vitro*, but may undergo metabolic activation to yield highly toxic metabolites. Mosquito toxicity of zone C compounds might not be enhanced by synergist treatment, and toxicity could even be decreased. If the activation process occurs preferentially in insects than humans, then this class of compounds would be selective to

insects. Compounds that do not have at least one of the values within the scale of this graph, are likely to be both a poor *Ag*AChE inhibitors and also non-toxic to mosquitoes. Since multiple processes may occur simultaneously *in vivo*, some compounds may fall between the zones. This model provides an approach whereby we can predict possible pharmacokinetic or pharmacodynamic processes that future compounds may undergo, based on inhibition and toxicity data.

4.4.2 Extended branching of the tert-*butyl group of PRC 331 and phenyl ring carbamoylation leads to loss of both selectivity and mosquito toxicity*

In this series of analogs, I explored mosquito-specific, phenyl-substituted selectophores of *N*-methylphenyl carbamates. Side chain branched analogs of PRC 331 show that complementarity to ACh, the AChE substrate, is a requirement for inhibition potency of our carbamates. Such a requirement has been historically observed with this group (Metcalf and Fukuto, 1965b). The *sec*-butyl moiety also causes a 13-fold gain in inhibition potency with HuAChE, an observation that clearly demonstrates the differential enzyme-ligand affinity between mosquito and human AChEs. A *tert*-pentyl group (PRC 540) gives unchanged potency and toxicity to mosquitoes; but shows substantial gain in HuAChE potency consistent with better steric fit. A 2-methylpentan-2-yl substituent (PRC 550) resulted in equi-potency between *Ag* and *Hu*AChE, but the toxicity to mosquitoes declined 8-fold. This may result from higher metabolic detoxication of PRC 550 compared to PRC 331 via hydroxylation of additional methyl groups, an assertion that can be confirmed by future PBO synergism studies.

Although the structural changes made in this series of compounds did not achieve superior anticholinesterase selectivity, I observed a consistent trend whereby all *tert*-butyl

signature compounds structurally similar to ACh (e.g., PRCs 331, 540 and 563), show similar *Ag*AChE inhibition potencies. However, PRC 521 and 550 were the best inhibitors of both AChEs, indicating that these two compounds have comparatively favorable interactions across both enzyme active sites while maintaining structural similarity to ACh. When the *sec*-butyl of PRC 521 and the 2-methylpentan-2-yl of PRC 550 project towards the anionic site, it creates favorable orientation of the side chains to interact through van der Waal's forces with this site, as was observed in a previous study by Metcalf and Fukuto (1962).

In a previous related study, inhibition of housefly AChE was maximal at 5 carbons in both *sec* and *tert* alkyl series (Kohn *et al*, 1965). From the current study, extended branching beyond 5 carbons did not cause substantial loss in *Ag* and HuAChE potency, although significant loss to mosquito toxicity was observed. The decrease in toxicity to mosquitoes consistent with extending the *tert*-butyl group of PRC 331 may implicate increased metabolic detoxication of these compounds, a subject of further investigation. Comparison of PRC 550 and 563 reveal that the spatial orientation of the side-chain in the enzyme anionic site determines the inhibition and consequently the selectivity of the analogs.

Hexyl substitution (PRC 507) meant to explore a methylene linkage strategy of a catalytic site ligand to a peripheral site moiety was unsuccessful. Carbamoylation of the phenyl ring (PRCs 565-569) while retaining the *tert*-butyl ring led to inactive compounds, where the relative lack of potency was higher with *Ag*AChE than HuAChE. This finding means that the mosquito enzyme has more 'strict' requirements for structural complementarity than the HuAChE.

4.4.3 Thioalkylation enhances mosquito selectivity but not toxicity, and bio-activation is an unlikely process in vivo

An *in vivo* activation of insecticides has been suggested as a strategy to achieve higher insect selectivity through selective metabolic bioactivation (Mahfouz *et al*. 1969; Metcalf, 1971). Bioactivation can involve the oxidation of a sulfur atom to more toxic sulfone or sulfoxide derivatives, *in vivo* (Staub *et al*. 1995, Distlerath, 1985). Thiolated carbamates were also shown to be more active on fly AChE than nonthiolated analogs ((Mahfouz *et al*. 1969). Mahfouz et al (1969) attributed the greater activity of alkylthiophenyl *N*-methylcarbamates to the polarization of the sulphur atom, whose partial positive charge mediate the higher affinity attraction to the AChE anionic site of fly AChE. Current data shows higher enzyme affinity with AgAChE than HuAChE, consequently leading to greater selectivity of some of the compounds in this series. Attempts to generate active di-substituted 2-thioalkyl,3-*tert*-butylphenyl N-methylcarbamates were unsuccessful.

By systematically extending the thioalkyl chain from ethyl-S (2 carbons, PRC 529) to 2-ethylbutyl-S (PRC 408), potency to *Ag*AChE, and not HuAChE, increased significantly. Toxicity to mosquitoes was optimal at C=4 (PRC 337), and declined with longer substituents. Longer or more elaborate alkyl branching led to a significant activity loss in toxicity to mosquitoes, consistent with similar findings by Mahfouz *et al* (1969). The potency and mosquito toxicity of PRCs 529, 530 and 337 to mosquitoes correlate, and therefore bioactivation was unlikely. However, contact toxicity of PRC 407 and 408 was low, despite both being highly potent to *Ag*AChE. Lack of enhanced *in vivo* toxicity via bioactivation of thio-substituted phenyl *N*-methylcarbamates has also previously been documented (Durden and Weiden, 1969; Nikles, 1969). Despite the successful use of sulfur-bioactivation strategy in drugs (Conti, 1977; Walsh, 2011) and some oxime carbamates (e.g.) aldicarb and methomyl (Felton, 1968; Gerolt, 1972,

Payne *et al*. 1966, Weiden *et al.* 1965), this approach has not worked with phenyl *N*-methylcarbamates based on current and previous findings.

4.4.4 Side chain halogenation had differential effects on toxicity and anticholinesterase activity

Fahmy *et al*. 1966 conducted a series of studies to investigate whether *in vivo* detoxication of *N*-methylcarbamates in insects occurred through P450-mediated hydroxylation of NCH$_3$ to NHCH$_2$OH. Their investigations involved testing synergized and non-synergized *N*-substituted analogs of *N*-methylcarbamates such as NHCD$_3$, NHCF$_3$, NH$_2$ and N-Si(CH$_3$)$_2$ derivatives. Related studies were also conducted by Widberg (1955), Cristol (1945), Kolbezen *et al*. (1954); and Dittert and Higuchi (1963). All these studies reported that the formation of NHCH$_2$OH is not the major detoxication pathway and that unmethylated (NH$_2$) carbamates were poor and unstable inhibitors. Consequently, metabolic detoxication in our series of compounds is likely to occur at the phenyl ring.

I explored the effect of halogenation on the superior toxicity of PRC 331 and related analogs. In these experiments, halogenated compounds were less toxic than their parent compounds and their AChE inhibitory potency did not correlate to toxicity, *in vivo*. For instance, PRC 515, which has a trifluorinated methyl placed on the *tert*-butyl moiety, displayed a 8-10 fold decrease in potency to both AChEs relative to parent compound (PRC 331), with little change in mosquito toxicity. Lack of either *Ag-* or HuAChE potency observed with PRC 515 maybe due to poor chemical complementarity to ACh. Presumably, the -CF$_3$ group increased toxicity by providing oxidative protection for the *whole tert*-butyl side chain, as observed in drug metabolism (Hughes *et al*. 2010; Müller *et al*. 2007; Park *et al*. 2001). Change of *tert*-butyl to a

CF$_3$ (PRC 505) caused complete insensitivity to both *Ag*- and HuAChE, but this compound was toxic to mosquitoes (LC$_{50}$ = 393 μg/mL). Its mode of action deserves further investigation.

4.4.5 Ring halogenation had differential effects on toxicity and anticholinesterase activity

Halogenation of the phenyl ring increases insecticidal activity with the trend *ortho > meta > para* substitutions (Hadaway and Barlow, 1965) and also with the trend I > Br > Cl > F (Metcalf *et al*. 1962b). The 6-bromo and 3-*tert*-butyl substituents of PRC 394 are at the optimal positions to confer the observed high AChE potency (Table 4.3). High *Ag*AChE potency of PRC 394 is not consistent with observed mosquito toxicity; hence, the presence of the bromo interfered with mosquito selectivity and toxicity. As expected, *ortho* bromination was favorable than *para* bromination of the phenyl group, and led to low activity of PRC 553. Bromination in PRC 394 did not yield the anticipated enhanced toxicity to mosquito, despite the gain in potency to *Ag*AChE. Halogenated carbamates have reduced hydrolytic stability due to the electron-withdrawing property of the halogen (Metcalf, 1971). It is therefore possible that halogenated compounds suffer degradation in the aqueous insect hemolymph before reaching the CNS.

Despite the high activity associated with *ortho* iodo halogenation (Metcalf, 1971), a di-substituted *ortho* iodo, *meta tert*-butylphenyl *N*-methylcarbamate (PRC 506) was inactive. Comparison of activity data for di-substituted compounds PRC 394 (*ortho* bromo) and PRC 506 (*ortho* iodo) reveals that AChE potency of halophenyl *N*-methylcarbamates does not depend solely on the position of halogens but also on the size of the halogen. Unlike the observations with mono-substituted halophenyl *N*-methylcarbamates (Metcalf *et al*. 1962b), halogenation at *ortho* in the presence of the *tert*-butyl group favors the smaller halogen. I also note that the potency change relative to size of the halogen was more pronounced in *Ag*AChE than HuAChE,

meaning that AChE structural differences may also play a role, presumably a more flexible and wider HuAChE active site tolerates a bigger halogen (*iodo*) compared to *Ag*AChE.

4.4.6 Trifluoromethylketone replacement of carbamate moiety

A non-carbamate trifluoromethylketone analog of PRC 331 (PRC 510) had low *Ag*AChE potency and mosquito toxicity, but was 210-times more potent to HuAChE (table 4.4). It was therefore the most non-selective in the series of compound tested. Trifluoromethylketones with a silyl side chain (PRCs 511 and 512) were of relatively low activity against both enzymes. Although inhibition of AChE by ketones is well documented (Dafforn *et al.* 1976 and 1982, Liu *et al.* 2007), no activity was observed against *Ag*AChE in current study. I only tested three compounds in this series, and it may be useful to explore further the basis of observed differences in AChE potency and strategies to improve mosquito selectivity and toxicity. The SAR of trifluoromethylketones is also relevant because studies have shown that electronegative substituents stabilize tetrahedral adducts formed from ketones thereby favoring hemiketal formation (Guthrie, 1975). The hemiketal is a structural analog to the tetrahedral intermediate formed in ester hydrolysis by AChE.

4.4.7 Silicon bearing alkyl substituents confer reduced AChE inhibition potency and mosquito toxicity than carbon bearing analogs

The greater ionic character of silico-carbon bond than the carbon-carbon bond (Rochow, 1951) prompted the AChE potency testing of the first series of trimethylsilylphenyl N-methylcarbamates with fly head AChE, by Metcalf *et al* (1962). In their study, the *meta* silyl substituent was shown to be the most active, 285-fold more potent than the unsubstituted. Hence

the silyl and alkyl substituents are both optimal at the *meta* position. Metcalf and co-workers also reported that trimethylsily analog was nearly half as potent to fly AChE, compared to the *tert*-butyl analog. In the current study, the trimethylsily compound (PRC 387) was significantly less potent to AChEs than the *tert*-butyl of PRC 331, consistent with previous data. This pattern of effects was generally true of all silicon-bearing compounds that I evaluated; hence carbamates with alkyl substituents are better AChE inhibitors and more toxic than their silyl isosteres. Thus, the greater length of the silicon-carbon bond (1.89 Å) compared to carbon-carbon bond (1.54 Å) (Rochow, 1951) was not an advantage, and may have violated the previously described critical spacing of esteratic and anionic interactants of carbamate AChE inhibition (Metcalf *et al*. 1962).

While I expected the greater ionic nature of $Si^{\partial+}$-$C^{\partial-}$ (Rochow, 1951) to enhance complementarity to the $-N^{+}(CH_3)_3$ of ACh and therefore greater inhibition potency, this is not certainly the case. The effects of silicon in this series of compounds was conserved in both AChEs, and were more superior than changes associated with alkyl branching, as evident from the minimal change in selectivity of silyl compounds when compared to PRC 331. Di-substitution by flourination (PRCs 559 and 547) or alkylation (PRCs 524, 525) of silylphenyl *N*-methylcarbamates was unfavorable, synonymous to data obtained with alkylphenyl analogs. Based on the unexpected toxicity of trifluoro-3-methylphenyl-*N*-methylcarbamate (PRC 505), activity of its silyl trifluorinated analog may provide valuable SAR.

4.4.8 Variants of PRC 472 are not potent anticholinesterases and non-toxic to mosquitoes

Initial conception of bivalent inhibitors included possible alkyl linkers of PRC331, such as PRC 507. However, lack of toxicity from alkyl substituted, t*ert*-butyl bearing compounds (e.g., 507) poses a challenge in designing of a bivalent carbamate whereby the linkage of PRC 331 to a peripheral active site ligand is required.

Another approach is the bivalent piperidine epitomized by PRC472, a compound having good enzyme selectivity, but low contact toxicity. Attempts to improve activity by chemical modification either reduced or eliminated inhibitory potency. Despite the disappointing results with this series, the excellent selectivity of PRC 472, a peripheral site ligand, provides us with a great opportunity to create a bivalent carbamate with inherent affinity to *Ag*AChE using different patters of chemical substitution.

References

Conti A., Bickel M.H. (1977). History of drug metabolism: discoveries of the major pathways in the 19th century. *Drug Metab. Rev*. 6:1–50.

Cristol S.J. (1945). A kinetic study of the dehydrochlorination of substituted 2,2-diphenylchloroethanes related to DDT. *J. Am. Chem. Soc.* 67: 1494-1498.

Dafforn A., Kerr P., and Murray R.R (1976). A ketopentyl transition state analog for acetylcholinesterase. *Biochem. Bioph. Res. Comm*. 73(2): 323-329.

Dafforn A., Neenan J.P., Ash C.E., Betts L., Finke J.M., Garman J.A., Mohan Rao M., Walsh K., and Williams R.R. (1982). Acetylcholinesterase inhibition by the ketone transition state analogs phenoxyacetone and 1-halo-3-phenoxy-2-propanones. *Biochem. Bioph. Res. Comm*. 104(2): 597-602.

Distlerath L.M., Loper J.C., and Tabor M.W. (1985) Metabolic activation of 3-(2-chloroethoxy)-1,2-dichloropropene: A mutagen structurally related to diallate, triallate, and sulfallate. *Environ. Mut*. 7(3): 303-312.

Dittert L.W., and Higuchi T. (1963) Rates of hydrolysis of carbamate and carbonate esters in alkaline solution. *J. Pharm. Sci.* 52: 852-857.

Durden J.A., and Weiden M.H.J. (1969) Insecticidal 2-(methylcarbamoyloxyphenyl)-1,3-dioxolanes, -oxathiolanesand –dithiolanes. *J. Agr. Food Chem.* 17: 94-100.

Ellman G.L., Courtney K.D., Andres V.J., and Feather-Stone R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmaco.l* 7: 88-95.

Fahmy M.A.H., Metcalf R.L., Fukuto T.R., and Hennessy D.J. (1966). Effects of deuteration, fluorination and other structural modifications on the carbamoyl moiety upon the anticholinesterase and insecticidal activities of N-methylcarbamates. *J. Agri. Food Chem.* 14: 79-83.

Felton J.C. (1968). Insecticidal activity of some oxime carbamates. *J. Sci. Food Agric*. 19: 32-38.

Feng S., Wang Z., He X., Zheng S., Xia Y., Jiang H., Tang X., and Bai D (2005). Bis-huperzine B: Highly potent and selective acetylcholinesterase inhibitors. *J. Med. Chem.* 48(3): 655-657.

Fridinger T.L., Mutsch EL, Bushong J.W., and Matterson JW (1971). Synthesis and biological activity of α-mercaptol O-(carbamoyl) oximes. *J. agric. Food Chem* 19(3): 422-431.

Gerolt P. (1972). The mode of entry of oxime carbamates into insects. *Pestic Sci.* 3: 43-55.

Guthrie J.P. (1975). Carbonyl addition reactions: Factors affecting the hydrate–hemiacetal and hemiacetal–acetal equilibrium constants. *Can. J. Chem*. 53(6): 898-906.

Gysin H., and Margot A. (1958). Chemistry and toxicological properties of O,O-Diethyl-O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate (diazinon). *J. Agric. Food Chem*. 6: 900-903.

Hadaway A.B., and Barlow F. (1965) The relative toxicity of adult mosquitos of derivatives of phenyl N-methylcarbamates. *Bull. World Health Org*. 32(4): 581-585.

Hughes L.M., Covian R., Gribble G.W., and Trumpower B.L. (2010). Probing binding determinants in center P of the cytochrome bc_1 complex using novel hydroxy-naphthoquinones. . *Biochim. Biophys. Acta*. 1797(1): 38-43.

Kohn G.K., Ospenson J. N., and Moore J.E. (1965). Some structural relationships of a group of simple alkyl phenyl N-methylcarbamates to anticholinesterase activity. *J. Agric. Food Chem.* 13(3): 232–235.

Kolbezen M.J., Metcalf R.L., and Fukuto T.R. (1954). Insecticide structure and activity, insecticidal activity of carbamate cholinesterase inhibitors. *J. Agric. Food Chem*. 2(17): 864–870.

Lee A.H., Sanborn J.R., and Metcalf R.L. (1973). Investigations into carbamate insecticide selectivity II: Differential degradation of selectophores in insect and mammal. *Pest. Biochem. Physiol*. 4: 77-85.

Lewis D.K. (1967). In vitro anticholinesterase activity of certain N-methylcarbamate insecticides compared with their N-acyl derivatives. *Nature* 1213(5072): 205-205.

Liu A., Guang H., Zhu L., Du G., Lee S.M., and Wang Y (2007). 3D-QSAR analysis of a new type of acetylcholinesterase inhibitors. *Chin. Life Sci*. 50(6): 726-30.

Mahfouz A.M., Metcalf R.L., and Fukuto T.R. (1969) Influence of the sulfur atom on the anticholinesterase and insecticidal properties of thioether N-methylcarbamates. *J. Agric. Food Chem.* 17(5): 917-922.

Metcalf R.L. (1971). Structure-activity relationships of carbamates. *Bull. World Health Organ*. 44(1-2-3): 43-78.

Metcalf R.L., and Fukuto T.R. (1965b). Silicon-containing carbamate insecticides. *J. Econ. Entomol.* 58(6): 1151-9.

Metcalf R.L., and Fukuto T.R., and Winton M.Y. (1962). Insecticidal carbamates: position isomerism in relation to activity of substituted phenyl N-methylcarbamates. *J. Econ. Entomol.* 55: 889-894.

Müller K., Faeh C., and Diederich F (2007). Fluorine in pharmaceuticals: looking beyond intuition. *Science* 317(5846): 1881-6.

Nikles E.F. (1969). *N*-methyl- and *N,N*-dimethylcarbamates of hydroxybenzaldehyde acetals and mercaptals. *J. Agric. Food Chem.* 17(5): 939-953.

Park B.K., Kitteringham N.R., and O'Neill PM. Metabolism of fluorine-containing drugs. *Annu. Rev. Pharmacol Toxicol.* 41: 443-470.

Pauling L. (1960). The nature of chemical bond and the structure of molecules and crystals. Cornell University Press, Ithaca NY. Pages 644-651.

Payne L.K., and Stansbury H.A. (1966). The Synthesis and Insecticidal Properties of Some Cholinergic Trisubstituted Acetaldehyde 0-(M ethylcarbamoy1)oximes. J. Agric. Food Chem. 14(4): 356-366.

Plimmer J.R. (2001). Handbook of pesticide toxicology, 2nd edtition. Robert I. Krieger and William C. Krieger (eds) pp 95-97.

Rochow E.G. (1951). An introduction to chemistry of silicons, 2nd ed. *John Wiley and Sons* Inc. NY.

Sanborn J.R., Lee A.H., and Metcalf R.L. (1973). Investigations into carbamate insecticide selectivity I: Evaluation of potential selectophores. *Pest. Biochem. Physiol.* 4: 67-76.

Staub R.E., Sparks S.E., Quistad G.B., and Casida J.E. (1995). S-methylation as a bioactivation mechanism for mono- and dithiocarbamate pesticides as aldehyde dehydrogenase inhibitors. *Chem. Res. Toxicol.* 8: 1063-1069.

Walsh J.S., and Miwa G.T. (2011). Bioactivation of drugs: Risk and drug Design. *Annu. Rev. Pharmacol. Toxicol.* 51:145-67.

Weiden M.H.J., Moorefield H.H., and Payne L.K. (1965). *0*-(methylcarbamoy1) oximes: a new class of carbamates insecticidesacaricides. *J. Econ. Entomol.* (58): 154-155.

Wiberg K.B. (1955). The deuterium isotope effect. *Chem. Rev.* 55(4): 713–743.

W.H.O. (2002a). WHOPES global insecticide use for vector-borne disease control. Geneva, World Health Organization. *WHO/CDS/WHOPES/2002.5*

W.H.O. (1981). Guidelines for determining the susceptibility or resistance of insects to insecticides. World Health Organization *WHO/VBC/81.806*, Geneva.

Chapter 5. Comparative Substrate Kinetics and Inhibition of Mosquito, Human and Chicken AChEs by Peripheral Site Ligands

Abstract

Recent molecular modeling suggests that selectivity of PRC 472 to AgAChE was due to preferential interactions with I70 in *Anopheles gambiae* acetylcholinesterase (AgAChE), which is Y70 in human (Hu) AChE. In order to test this model, several *in vitro* experiments were conducted. AChE gorge mapping with *bis*(n)-tacrine dimers showed that gorge geometry, as measured by sensitivity to these inhibitors for AgAChE-I70Y and HuAChE was more similar for these enzymes than AgAChE wild type. The AgAChE-I70Y, as well as AgAChE (recombinant and homogenate), chicken (Chk) AChE and HuAChE were tested against other known peripheral site ligands, namely: propidium, BW284c51 and ethidium, and the new mosquito-specific ligand, PRC 472. BW284c51 was the most potent ligand to all AChEs. Potency of PRC 472 to AgAChE-I70Y decreased significantly (44-fold) when compared to AgAChE-LiN, indicating that selectivity of this ligand to AgAChE was abolished by the I70Y mutation. Both propidium and ethidium were more potent to HuAChE and AgAChE-I70Y than all other AChEs. Substrate specificity and enzyme kinetics of AChEs was evaluated with acetylthiocholine-iodide (ATChI) as substrate, to generate Km and Vmax values. Km values of AgAChE-I70Y and HuAChE were not significantly different. Since the I70Y did not affect the Vmax, the decrease in affinity to substrate associated with this mutation does not affect catalytic efficiency of the enzyme. The three indices; gorge geometry, inhibition by ligands, and substrate kinetics are all in support of the proposed model that selectivity of PRC 472 to AgAChE is due to its preferential interactions with I70, which is Y70 in HuAChE.

5.1 Introduction

Early studies revealed that AChE has two distinct ligand binding sites involved in either activation or inhibition of substrate catalysis (Changeux, 1966). Since then numerous studies have mapped the two sites and their functional importance. Peripheral anionic site (PAS) inhibitors have been shown to affect substrate catalysis through mechanisms such as steric blockade of ligands into and out of the gorge, and also by allosteric effects at the catalytic anionic site (Berman et al. 1981; Taylor and Lappi 1975; Szegletes et al. 1998; Colletier et al. 2006). As more information about the nature of these two unique sites became available, the design of dual binding ligands ensued. This led to new drugs for Alzheimer's disease (AD), such as the highly potent and AChE-selective E2020, as well as bivalent derivatives of Huperzine A, among others (Nightingale 1997; Wong et al. 2003; and a review by Haviv et al. 2007).

The great efficacy of E2020 was attributed to its high selectivity towards AChE, compared to butyrylcholinesterase (BuChE), and consequently fewer side effects to AD patients (Thomsen and Kewitz, 1990; Cheng et al. 1996; Galli et al. 1994). Further analysis revealed that selectivity of E2020 to AChE is due to interaction with sites that are unique and specific to AChE, and not found in BuChE (Loewenstein et al. 1993; Harel et al. 1992). Despite the achievement of bivalent ligands for use in medicine, such has not been the case in the search of selective bivalent insecticides. Although differences between vertebrate and insect acetylcholinesterase have been widely studied (Carlier et al. 2008; Pang et al. 2009; Anderson et al. 2008; Mutunga et al. 2009; Polsinelli et al. 2010), the success in discovering specific selective ligands has been low.

Along these lines, the presence of I70 in AgAChE (HuAChE has Y70) led to loss of π-stacking interactions with bis(7) tacrine, attributable to low potency to AgAChE, when compared to HuAChE (Carlier et al. 2008). Recent molecular modeling studies performed by our

collaborators Max Totrov and Polo Lam (Molsoft LLC, La Jolla, CA) reveal that the high mosquito-selectivity of PRC 472, an E2020-inspired derivative is due to preferential interaction of the ligand with I70 (Fig. 5.6A-C). Such differences in amino acid content are important targets for design of ligands that specifically interact with residues in mosquito that are absent in human enzyme, and vice versa.

In pursuit of further validating this model, I performed systematic gorge mapping of selected AChE phenotypes using dimeric tacrines with varying tether lengths as structural probes. Enzymes used in this study were wild-type (WT) human recombinant AChE (HuAChE), recombinant *An. gambiae* AChE catalytic domain (*Ag*AChE-LiN) and mutant recombinant *Ag*AChE whose I70 is substituted with Y70, to mimic the human phenotype (*Ag*AChE-I70Y). I also used mosquito homogenates of G3-strain (*Ag*AChE-G3; susceptible) and Akron-strain (*Ag*AChE-G119S; resistant) as enzyme sources. Chicken brain homogenate was used as a source of ChkAChE. The chicken enzyme is a "natural triple mutant" since it lacks three key PAS residues namely Y70, Y121 and W279 (Eichler et al. 1993). In addition to the gorge mapping studies, I also evaluated the inhibition potency to AChEs of (bis) quaternary compounds and specific peripheral site ligands (PSLs); namely, BW284c51, PRC 472, propidium, and ethidium. Additionally, enzyme-substrate kinetic studies with acetylthiocholine (ATCh) were performed with some of the enzyme species. Results from both AChE gorge geometry and inhibition potency provide plausible evidence that the I70Y mutation in *Ag*AChE confers a more 'human' phenotype to the mosquito enzyme. A comparative account of the differential effects of the PSLs with enzyme phenotypes is also provided.

5.2 Materials and methods

5.2.1 Chemicals, reagents and enzymes

Tacrine, propidium iodide, ethidium bromide and BW284c51 (Fig. 5.1) were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA). PRC 472 and *bis*(n)-tacrine dimers (n = 2, 3, 4, 5, 6, 7, 8, 9, 10, and 12 methylenes) were synthesized and purified to > 95% at the laboratory of Dr. Paul Carlier, Department of Chemistry, Virginia Tech (Fig. 5.1).
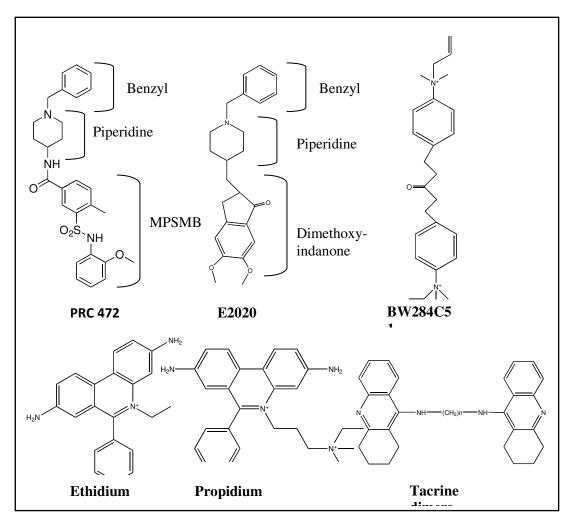
Figure 5. 1. Structures of AChE inhibitors used in this study. The lower portion of PRC 472 that binds to the PAS is a methoxyphenyl sulfamoyl-4-methylbenzamide, and is abbreviated as MPSMB, for ease of reference. For the tacrine dimers, n= 2, 3, 4, 5, 6, 7, 8, 9, 10, and 12 methylene units.

All buffer components and HuAChE were purchased from Sigma, as above. Recombinant mosquito enzymes *Ag*AChE-LiN (N= normal, representative of WT recombinant *Ag*AChE) and *Ag*AChE-I70Y were supplied by Dr. Jianyong Li, of the Department of Biochemistry, Virginia Tech. Recombinant double *Ag*AChE mutant M438P and D441Y and a single *Ag*AChE-F329S supplied by Dr. Li were not catalytically active, and therefore could not be functionally characterized. I used whole Akron and G3 mosquitoes, and chicken brain homogenates as sources of *Ag*AChE-G119S (Akron AChE), *Ag*AChE-G3 and ChkAChE, respectively. The two mosquito strains G3 (MRA-112) and Akron (MR4-913 deposited by Akogbeto and possess both *ace*-1 G119S and *kdr* L1014F resistance mutations), were maintained at the Fralin Insectary, Department of Entomology, whereas chicken brain was donated by Dr. Marion Ehrich, of the Department of Biomedical Sciences & Pathobiology, Virginia Tech.

5.2.2 AChE inhibition assays

The Ellman method (Ellman *et al*. 1961) was used to determine residual AChE activity values for the tacrine dimers as described in detail in chapter 2, without modifications. The same method was used to generate residual AChE activity values for peripheral site ligands, as detailed in chapter 4, without modifications. Residual AChE activity values were expressed as percent of control and plotted in Prism (GraphPad Software, San Diego, CA, USA) to generate IC_{50} values, 95% confidence intervals (CI), etc.

5.2.3 *Substrate kinetics assay and AChE quantification*

Quantification of recombinant *Ag*AChE I70Y was performed using the Pierce Micro® BCA protein quantification kit (Thermo Scientific, Rockford, IL, USA) as per manufacturer's instructions. This was done in order to assess the yield of *Ag*AChE-I70Y and for any future comparisons with the recombinant *Ag*AChE-LiN. Total protein quantified for the stock of recombinant *Ag*AChE-I70Y supplied was 9.8 mg/mL. For the Ellman assays (inhibition potency and substrate kinetics), the stock was diluted 500-fold dilution onto working solution and a further 20-fold dilution on the Ellman plate, hence the final protein content in *Ag*AChE-I70Y was 0.98 µg/mL. This concentration was optimized to give a reaction rate of 0.04 OD/min, the rate that was standardized for all the AChEs in Ellman assay reactions, at 0.3 mM of DTNB and 0.4 mM of ATChI.

The substrate used in this study was acetylthiocholine iodide (ACThI) at five concentrations (0.0625 to 2 mM) using the Ellman reaction. Enzyme activities were determined spectrophotometrically using a Dynex® 96-well plate reader (Dynex Technologies, Chantilly, VA) with 0.4 mM of DTNB and monitored every 17 sec over a 3 min period, at 25 ^{0}C and 405 nm.

$$\text{AChE Activity (U)} = \frac{(\text{OD/min/1000}) \times 10^6\,\mu\text{Mmin}^{-1}\text{mL}^{-1} \times \text{dil. factor}}{\text{DTNB Extinction Coefficient}^a \times \text{path length}^b}$$

[a]Extinction coefficient of DTNB at 405 nm and 25 ^{0}C $= 12800$ M^{-1}cm^{-1}
[b]Path length $= 0.5$ cm, with 200 µL per well.

The Michaelis-Menten constant (*K*m, Eqn. 5.2) and maximal velocities (Vmax) were determined by non-linear regression analysis of specific activity (units) with substrate concentration in micromolar, to generate the kinetic plots (Fig. 5.4) in Prism®.

$$\text{Michaelis-Menten equation: } V_o = \frac{Vmax * [S]}{(Km + [S])}$$

V_o and Vmax are the initial and final enzyme velocities; respectively, and [S] is the substrate concentration. *K*m is the substrate concentration at half of Vmax, and is a Michaelis-Menten constant for every substrate and enzyme.

Quantification of recombinant *Ag*AChE I70Y was performed using the Pierce Micro® BCA protein quantification kit (Thermo Scientific, Rockford IL) as per manufacturer's instructions. This was done in order to assess the yield of *Ag*AChE-I70Y and for any future comparisons with the recombinant *Ag*AChE-LiN.

5.3 Results

5.3.1 Inhibition of AChEs by dimeric tacrines

Inhibition potency of the following AChEs with inhibitors THA and dimeric tacrines was evaluated: AgAChE-I70Y, AgAChE-G119S and ChkAChE, as shown in Table 5.1 below.

Table 5. 1. IC_{50}s of tacrine dimers with mutant mosquito AChEs and Chicken AChE.

Inhibitor	Enzyme species; IC_{50}, nM (95% CI)		
	AgAChE-G119S	AgAChE-I70Y	ChkAChE
THA	435 (372-508)[a,1]	1571 (1473-1676)[a,2]	41 (37-47)[a,3]
bis(2)-tacrine	1486 (1276-1731)[b,1]	3164 (2955-3387)[b,1]	1255 (1093-1442)[b,1]
bis(3)-tacrine	399 (369-432)[a,1]	469 (416-529)[c,1]	925 (865-990)[c,2]
bis(4)-tacrine	599 (539-665)[c,1]	129 (125-133)[d,2]	134 (127-142)[d,2]
bis(5)-tacrine	390 (348-438)[a,1]	75 (73-77)[e,2]	60 (56-65)[e,3]
bis(6)-tacrine	156 (139-176)[d,1]	71 (67-75)[e,2]	34 (32-36)[f,3]
bis(7)-tacrine	56 (50-62)[e,1]	26 (24-28)[f,2]	96 (87-107)[g,3]
bis(8)-tacrine	28 (25-32)[f,1]	26 (25-28)[f,1]	44 (42-47)[a,2]
bis(9)-tacrine	58 (52-65)[e,1]	15 (13-17)[g,2]	124 (118-134)[d,3]
bis(10)-tacrine	99 (93-106)[g,1]	42 (39-45)[h,2]	131 (108-160)[d,3]
bis(12)-tacrine	375 (342-412)[a,1]	240 (222-259)[i,2]	147 (123-180)[d,3]

IC_{50} values in columns or rows bearing the same superscript letter or number; respectively, are statistically not significant, based on overlapping 95% CI ($P > 0.05$).

135

Inhibition potency of *bis(2)*-tacrine was the lowest overall, and the differences in IC_{50} values were not statistically significant among the three AChEs. Inhibition potency to *Ag*AChE-G119S, *Ag*AChE-I70Y and ChkAChE was highest with *bis*(8)-, *bis*(9)- and *bis*(6)-tacrines, respectively. Overall, the difference between the least and most potent inhibitor was highest in *Ag*AChE-I70Y (211-fold), followed by *Ag*AChE-G119S (53-fold), and lowest in ChkAChE (37-fold). Likewise, the tether length-dependent potency gain between the monomer and most potent dimer was, 105-fold in *Ag*AChE-I70Y, 15-fold in *Ag*AChE-G119S, and 1.2-fold in ChkAChE.

Potency data in Table 5.1 was plotted together with other published data as shown in Fig. 5.2, in order to depict the trend of inhibition by dimeric tacines, across different AChEs. Overall, low IC_{50} values were observed with HuAChE, followed by *Ag*AChE-I70Y, whereas *Ag*AChE WT had the highest IC_{50}s. Note also that the mutation of I70Y in *Ag*AChE changes the pattern of responses with *bis*(n)-tacrines to more closely match that of HuAChE (Fig. 5.3). Longer tethers (i.e.) *bis*(10-12) show a loss in potency in all species except chicken, whose potency remains similar at *bis*(9-12), creating a 'plateau' region. For each case, either THA or *bis*(2)-tacrine was the least potent inhibitor. Maximal potency of all AChEs is observed between *bis*(6-9)-tacrines, a three methylene linker range.
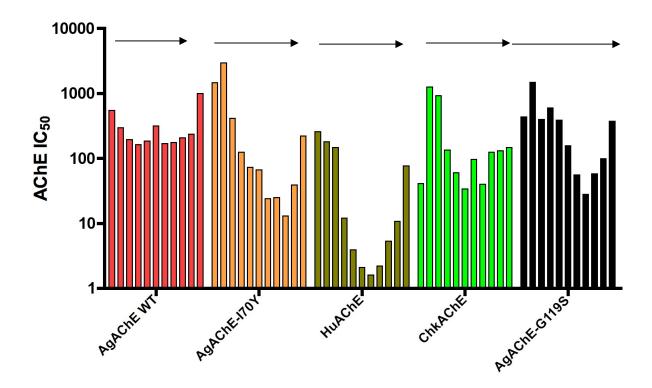
Figure 5. 2. Comparative potency of dimeric *bis*(n)-tacrines showing AChE-IC$_{50}$s (nM). Potency data for *Ag*AChE-WT (G3 mosquito homogenate) and HuAChE were adapted from Anderson et al, (2008). Arrows on top of each panel indicate direction of increasing tether lengths from the tacrine monomer (left most bar in each set), progressing through n = 2, 3, 4, 5, 6, 7, 8, 9, 10, to bis(12) tacrine (right most bar).

5.3.2 Inhibition of AChEs by peripheral site ligands

Evaluation of inhibition potency of PSLs (Fig. 5.3, Table 5.2) was done with native Akron mosquito (*Ag*AChE-G119S), G3 mosquito (*Ag*AChE-WT), and chicken brain (ChkAChE) homogenates, and three recombinant AChE types namely; mosquito WT enzyme (*Ag*AChE-LiN), *Ag*AChE-I70Y, and HuAChE. The enzymes tested have different structural motifs at both the peripheral (e.g., *Ag*AChE-I70Y) and catalytic sites (e.g., *Ag*AChE-G119S).

137
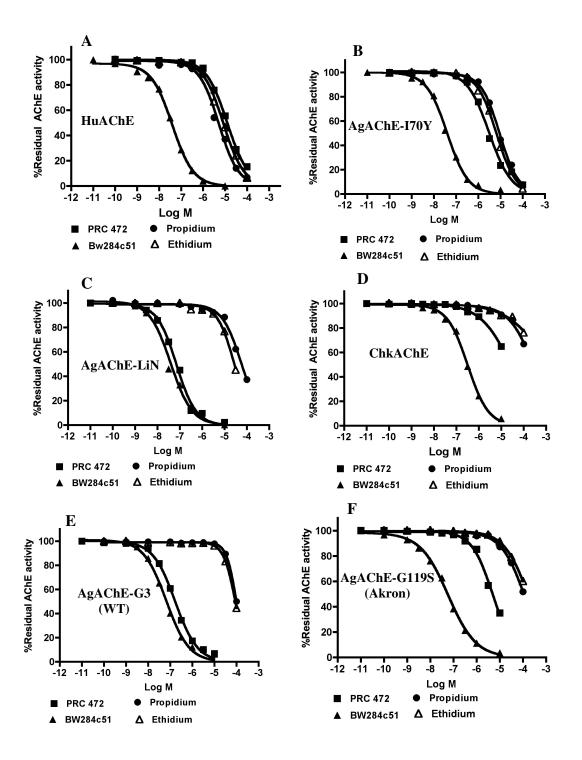
Figure 5. 3. Inhibition potency of peripheral site ligands to AChEs. Letters denote: HuAChE (**A**), *Ag*AChE-I70Y (**B**), *Ag*AChE-LiN (**C**), ChkAChE (**D**), WT *Ag*AChE-G3 (**E**) and Akron *Ag*AChE-G119S (**F**).

In Fig. 5.3, similar inhibition patterns are observed between HuAChE and *Ag*AChE-I70Y, and also between *Ag*AChE-LiN and WT *Ag*AChE-G3. Although *Ag*AChE-G119S was more sensitive to PRC 472 and BW284c51 than ChkAChE, their relative inhibition patterns were quite similar. BW284c51 was the most potent inhibitor in all four AChEs, whereas the least potent ligand varies across AChEs. Of all the AChEs, ChkAChE was the least sensitive to the PSLs. The IC_{50}s generated by sigmoidal plots in Fig 5.3 were presented in Table 5.2 for statistical comparison. Potency of PRC 472 to *Ag*AChE-LiN was 44-fold higher than in *Ag*ACHE-I70Y, whereas the difference between *Ag*AChE-I70Y and HuAChE was 4-fold (Table 5.2). Potency of BW284c51 to G3 and Akron AChEs is similar, but potency of PRC 472 decreases ca. 30-fold in Akron AChE compared to G3. Specifically, potency of PRC 472 to *Ag*AChE-G119S is ca. 85-fold lower than that of BW284c51, while the difference in *Ag*AChE-G3 (WT) is < 3-fold. ChkAChE was least sensitive among all the enzymes to BW284c51 and was inhibited with low potency by PRC 472, but both propidium and ethidium were inactive. Propidium and ethidium are both poor inhibitors of G3 and Akron AChE, and more potent (ca. 2-fold) to HuAChE than PRC 472. The profile is reversed for *Ag*AChE-I70Y, where PRC 472 is about 2-fold more potent as an inhibitor than propidium or ethidium (Table 5.2).

Table 5. 2. Inhibition potency of peripheral site ligands to AChEs used in this study.

AChE Source	Inhibitor IC$_{50}$, nM (95% CI)			
	PRC 472	BW284c51	Propidium	Ethidium
HuAChE	12,470 (11800-13180)[a,1]	39 (36-42)[a,2]	5,124 (4512-5819)[a,3]	8,229 (7623-8882)[a,4]
*Ag*AChE-I70Y	3,046 (2668-3479)[b,1]	34 (32-37)[a,2]	8,283 (7595-9634)[b,3]	6,472 (6173-6785)[b,4]
*Ag*AChE-LiN	70 (63-78)[c,1]	39 (32-47)[a,2]	58,480 (53490- 63920)[c,3]	27,840 (25840-30010)[c,4]
ChkAChE	26,540 (24180- 29130)[d,1]	318 (297-341)[b,2]	>100,000; 67% RAR*	>100,0000; 76 % RAR*
WT *Ag*AChE-G3	166 (152-182)[e,1]	66 (60-73)[c,2]	100,080 (99440-102200)[d,3]	88,110 (85640-90650)[d,4]
*Ag*AChE-G119S	4,904 (4565-5268)[f,1]	58 (54-63)[c,2]	>100,000; 52% RAR*	>100,000; 60% RAR*

*RAR denotes Residual Activity Remaining, at 100, 000 nM; the highest inhibitor concentration tested. IC50 values in columns or rows bearing the same superscript letter or number; respectively, are statistically not significantly different, based on overlapping 95% CI (P > 0.05).

5.3.3 Substrate specificity and enzyme kinetics of acetylcholinesterases

The Km and Vmax values were determined for four enzyme phenotypes; HuAChE, *Ag*AChE-I70Y, *Ag*AChE-LiN and ChkAChE. Enzyme activity was measured in the presence of ATChI as a substrate, and plotted in Prism® to generate the graph in Figure 5.4. The enzyme activity of *Ag*AChE-LiN and *Ag*AChE-I70Y follow a similar trajectory with ATChI. There is a significant increase in maximal activity of ChkAChE with ATChI, when compared to other enzymes. ATChI had the highest Vmax with Chicken, followed by mosquito and lastly human AChE (Table 5.5). Lowest Km value for ATChI was obtained with *Ag*AChE-LiN followed by ChkAChE, HuAChE and *Ag*AChE-I70Y, in that order.
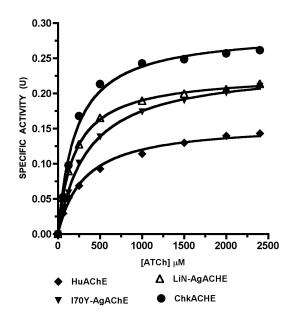

Figure 5. 4. Substrate velocities of AChEs with ATChI. All enzyme quantities used were standardized to give a rate of 0.04/OD per min over 10 min, which is the optimized rate for first-order kinetics, at 0.4 mM of ATChI.

141

Table 5. 3. Kinetic properties of human, mosquito and Chicken AChEs

Enzyme	ATChI	
	Km; μM	Vmax; U
HuAChE	311 (237-385)[a,c]	0.158 (0.148-0.168)[a]
AgAChE-I70Y	358 (327-389)[a]	0.238 (0.232-0.244)[b]
AgAChE-LiN	199 (185-213)[b,d]	0.228 (0.224-0.232)[b]
ChkAChE	211 (159-262)[c,d]	0.289 (0.272-0.306)[c]

Values in brackets are 95% CI, and are from means of four replicates. Values of kinetic parameters in the same column, followed by same superscript letter are not significantly different, based on their overlapping 95% CI (P < 0.05).

5.3.4 Important amino acids for ligand docking of PRC 472 inferred from AChE sequence analysis and a molecular model of Y70/Y71 with docked PRC 472

A multiple linear sequence alignment of vertebrate and invertebrate AChEs was generated using Clustal W (Fig. 5.4). The missing aromatic residues typically implicated as functionally important for ligand binding are denoted by letters **a**, **b** and **c**. The **'a'** denotes the Y70 in HuAChE, which is M70 in Chicken (Gallus) and I70 in both *Blattella germanica* and *Anopheles gambiae*. The **'b'** denotes the Y121 in HuAChE, which is T121 in chicken. The second residue to the left of Y121 is the G119, which is S119 in Akron mosquitoes (*ace*-1 resistance to carbamates). The **'c'** denotes W279 in HuAChE, which is G279 in chicken. For consistency, all residue numbering is based on *Tc*AChE sequence.

```
         a
Homo       G---VVDATTFQSVCYQYVDTLYPGFEGTEMWNPNRELSEDCLYLNVWTPYPRPTSPTPV 114
Bos        G---VLNATAFQSVCYQYVDTLYPGFEGTEMWNPNRELSEDCLYLNVWTPYPRPSSPTPV 111
Rattus     G---ILDATTFQNVCYQYVDTLYPGFEGTEMWNPNRELSEDCLYLNVWTPYPRPTSPTPV 114
Gallus     G---IRDADSQPFACYQMVDTTFPGFQGSEMWNPNREMSEDCLYLNVWTQKGDPTEP-PV 116
Torpedo    G---VWNASTYPNNCQQYVDEQFPGFSGSEMWNPNREMSEDCLYLNIWVPSPRPKST-TV 111
Anopheles  G---VLNTTTPPNSCVQIVDTVFGDFPGATMWNPNTPLSEDCLYINVVAPRPRPKNA-AV 111
Blattela   KHGEIYNATKMPNSCVQIIDTVFGDFPGALIWNPNTPLSEDCLYINVVVPKPRPKNA-AV 114

                      b
Homo       LVWIYGGGFYSGASSLDVYDGRFLVQAERTVLVSMNYRVGAFGFLALPGSREAPGNVGLL 174
Bos        LVWIYGGGFYSGASSLDVYDGRFLVQAEGTVLVSMNYRVGAFGFLALPGSREAPGNVGLL 171
Rattus     LIWIYGGGFYSGASSLDVYDGRFLAQVEGTVLVSMNYRVGTFGFLALPGSREAPGNVGLL 174
Gallus     LVWIYGGGFTGGSVSLDVYDGRYLAAAEEAVVVSMNYRVGSLGFLALAGHRDAPGNVGLW 176
Torpedo    MVWIYGGGFYSGSSTLDVYNGKYLAYTEEVVLVSLSYRVGAFGFLALHGSQEAPGNVGLL 171
Anopheles  MLWIFGGGFYSGTATLDVYDHRALASEENVIVVSLQYRVASLGFLFLG-TPEAPGNAGLF 170
Blattela   MVWIFGGGFYSGSATLDVYDHKTLVSEENVIIVSMQYRVASLGFLFFD-TIDVPGNAGLF 173

                                                              c
Homo       PWATVGMGEARRRATQLAHLVGCPPGGTGGNDTELVACLRTRPAQVLVNHEWHVLPQESV 294
Bos        PWATVGVGEARRRATLLARLVGCPPGGAGGNDTELVACLRARPAQDLVDHEWRVLPQEHV 291
Rattus     PWATVSAGEARRRATLLARLVGCPPGGAGGNDTELISCLRTRPAQDLVDHEWHVLPQESI 294
Gallus     PWATIGAAEGRRRAAALGRAVGCPYG----NETEFLGCLRGKEAADVLEGEGVVMPPQSV 292
Torpedo    PWASVSVAEGRRRAVELGRNLNCNLN----SDEELIHCLREKKPQELIDVEWNVLPFDSI 287
Anopheles  PWALVSREEATLRALRLAEAVGCPHEPS--KLSDAVECLRGKDPHVLVNNEWGTLG---I 285
Blattela   PWAIISREESILRGLRLAEAVGCPRSRS--DIRAVIDCLRKKNATDLVNNEWGTLG---I 288
```

Figure 5. 5. Excerpts of multiple sequence alignments of AChE protein sequences by Clustal W. Letters on top of each panel refer to the residues vertical to them and denote functionally important residues as described in the text.

The models in Fig 5.6 (A-C) were generated by Dr. M Totrov and Lam P.C.H. of Molsoft L.L.C. In Fig 5.6A, the aromatic side chain of Y70 in HuAChE occupies the pocket that the phenyl ring of PRC 472 fits into and therefore binding of the ligand is compromised. In Fig 5.6B, the binding pocket of PRC 472 is not occluded by the smaller side chain of I70, and therefore the ligand favorably binds to the mosquito enzyme. Figure 5.6C shows PRC 472 docked onto *Ag*AChE, with its phenyl ring occupying the pocket next to I70 (in bold). The faintly visible Y121 is at the back of the gorge side chain projects to a different panel and does not occlude the binding pocket of PRC 472; consequently, Y121 does interfere with binding of the ligand.
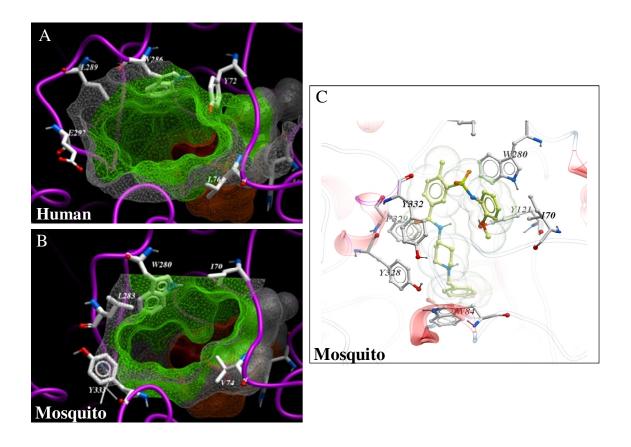
Figure 5. 6. Differences in molecular space occupied by Y70 (A) and I70 (B) at the peripheral site of AChE and a molecular model of PRC 472 docked into mosquito AChE (C).

5.4 Discussion

Highly conserved aromaticity of the active site gorge (Gentry and Doctor, 1991), coupled with numerous studies including computer docking, structural complexes of AChE-ligand complexes and site-directed mutagenesis, all confirm a functional role of such residues to AChE (Sussman et al. 1991, 1992; Harel et al. 1992, 1993; Vellom et al. 1993; Shafferman et al. 1992). Molecular modeling reveals that Y70, Y121 and W279 are functionally important for the peripheral site of HuAChE and *Torpedo* AChE (Radic

144

et al. 1994). Linear sequence alignments (Fig 5.5) show some naturally lacking, functionally important homologous aromatic residues pertinent to my work.

The *Ag*AChE selective ligand PRC 472, which is structurally similar to E2020, represents a mosquito-selective ligand that can be used in the design of selective bivalent insecticidal carbamates. Due to the high relatedness in the structure of E2020 and PRC 472, I briefly highlight some functional properties of the well-studied E2020 compound. The high activity of E2020 (Aricept®) to AChE is attributed to a series of interactions of the benzyl, piperidine and indanone moieties to the CAS, the 'bottle-neck', and PAS residues, respectively (Kryger et al. 1999). Specifically, the benzyl group interacts via π-π stacking with the indole moiety of W84 at the choline-binding (anionic) site, similar to the interaction observed with tacrine (Kryger et al. 1999; Harel et al. 1993; Harel et al. 1996). At the gorge bottleneck, cation-π interactions between the cationic nitrogen of the piperidine and phenyl ring of F330 close to the anionic site, as well as water mediated H-bonding with Y121 closer to the PAS, all stabilize the ligand binding process (Inoue et al. 1996, Verdonk et al. 1993; Dougherty, 1996).

At the peripheral anionic site (PAS), the indanone ring stacks together with the indole ring of W279 via π-π interaction. Hydrogen bonds between the methoxy group of E2020 and both a water molecule E185, also further stabilize this interaction (Kryger et al. 1999). The carbonyl group of indanone interacts via Van der Waals forces with aromatic rings of F331 and F290 (Cardozo et al. 1992). These interactions help to constrain E2020 in a favorable orientation whereby the indanone group interacts with W279 (Kryger et al. 1999). The structural relatedness of E2020 to PRC 472 may suggest that the latter mediates its potency and selectivity in a similar manner. However, the

greater selectivity of PRC 472 to mosquito AChE, compared to human indicates that more favorable mosquito-specific interactions occur between the ligand and *Ag*AChE.

A molecular model by Molsoft (Fig. 5.6A-C) suggests that the mosquito-selectivity of PRC 472 is due to a better fit of PRC 472's phenyl moiety to a binding pocket adjacent to I70 in the mosquito (Fig 5.6B). This binding pocket is narrower in HuAChE due to the presence of the side chain of Y70, therefore the phenyl ring of PRC 472 does not fit into the binding pocket (Fig 5.6A) leading to low potency of the ligand to this enzyme. Both *Ag* and HuAChE possess a conserved Y121. Although Y121 is visible in the model (Fig 5.6C), it is at the back of the gorge and its side chain does not occupy the binding pocket of PRC 472. The model suggests that I70 in *Ag*AChE allows for better fit of PRC 472, whereas the space is narrower due to the presence of Y70 in HuAChE, consequently leading to lower potency of PRC 472 to HuAChE. In order to test this hypothesis, a recombinant *Ag*AChE-I70Y mutant was generated to mimic the PAS architecture of HuAChE. Additional studies of inhibition by PSLs of other 'natural' mutants, namely the ChkAChE, which lacks Y70, Y121 and W279, are reported. As expected, in both cases the enzyme had low affinity for PRC 472, confirming the model.

5.4.1 The gorge-geometry of Ag*AChE-I70Y resembles that of HuAChE, and ChkAChE has a unique plateau region at longer tethers*

AChE potency was lowest at THA and *bis*(2)-tacrine across all enzymes, synonymous with a structurally conserved CAS at the bottom of the AChE gorge. However, it is unclear why THA was more potent to ChkAChE, compared to all other AChE species. Maximal potency to all AChEs was between *bis*(6-9)-tacrines,

synonymous with a high level of similarity in the AChE gorge across phenotypes. The same trend was observed with human, bovine and rat AChEs, where highest potency was observed between *bis*(5)-tacrine and *bis*(7)-tacrine (Anderson et al. 2008; Muñoz-Ruiz et al. 2005; Wang et al. 1999) Comparison of HuAChE and the *Ag*AChE-I70Y shows a greater resemblance in gorge geometry resulting from Y70, evident from the tacrine dimer plots (Fig. 5.3). This resemblance is also observed to some extent in *Ag*AChE-Akron (G119S), but not with G3 *Ag*AChE WT. A striking feature of the Akron AChE is the subtle gain in potency with THA up to *bis*(5)-tacrine, as opposed to equivalent greater potency enhancement with all other AChEs. Quantitatively, net potency gain between THA and *bis*(5)-tacrine for *Ag*AChE-G119S was ca. 4-fold, compared to 42-fold and 21-fold gain for *Ag*AChE-I70Y and ChkAChE, respectively (Table 5.1). Observed low potency gain could result from the occlusion and crowding of the CAS by the bulkier side chain of serine in *Ag*AChE-G119S, as opposed to that of glycine in un-mutated catalytic sites.

The maximal potency gain, obtained by difference between THA and most potent dimer, decreased in the following order: HuAChE > *Ag*AChE-I70Y > *Ag*AChE-G119S > ChkAChE > *Ag*AChE WT. This trend shows another close association of HuAChE with *Ag*AChE-I70Y. There is no clear explanation of the differences in net potency gain observed between *Ag*AChE-G119S and WT *Ag*AChE, and the G119S mutation could not be directly implicated. The geometrical relatedness observed at longer tethers across all AChEs except ChkAChE indicates that although variations in inhibitory potency occur at such long tethers, the pattern of inhibition remains similar. Unlike all other AChEs, the geometry of ChkAChE shows a 'plateau region' at longer tethers, characteristic of unique

interaction of dimeric tacrines at the peripheral site. The 'plateau region' indicates that the 'steric' clash at the PAS, associated with the other AChEs does not occur with ChkAChE. This results possibly because of the natural mutants Y70M, Y121T and W279G in ChkAChE, all of which introduce less aromaticity, while maintaining H-bonding capacity. Another explanation could be that ChkAChE peripheral site is less constricted than for other AChEs, and after a certain tether length, few additional specific interactions occur. AChE multiple sequence alignment reveals a unique 112 amino acid fragment inserted at the N310 residue of TcAChE (data not shown). Since there are no currently available crystal structures or molecular models for ChkAChE, I am unable to neither detail the structure of PAS nor explain the possible role of the 112-residue fragment in function or gorge geometry of ChkAChE.

5.4.2 The I70Y mutation abolished selectivity of PRC 472 to AgAChE, and BW284c51 is a non-selective AChE peripheral site ligand

IC$_{50}$ values for all PSLs with the five AChEs were significantly different (P < 0.05; Table 5.2), indicating that all PSLs inhibited each AChE differently; except for the unquantifiable inhibition of ChkAChE, *Ag*AChE-G3 and *Ag*AChE-G119S by propidium and ethidium. Interestingly, BW284c51 is equipotent to the 3 enzyme phenotypes; HuAChE; *Ag*AChE-I70Y and *Ag*AChE-LiN, with only a small difference in potency of BW284c51 with mosquito homogenates (G3 and Akron AChEs) and HuAChE. Previous findings by Jiang et al (2009) show that BW284c51 had an IC$_{50}$ of 199 nM with a recombinant AgAChE, which presents a ca. 5-fold difference compared to our data with *Ag*AChE-LiN. The difference can be attributed to the fact that AChE purity in these

studies was different, therefore further characterization of enzyme kinetics with purified *Ag*AChE-LiN is underway. In a related study, Zhao *et al.* (2010) reported a BW284c51 IC_{50} of 1660 nM with recombinant AChE of the greenbug Schizaphis graminum. It is unlikely that the > 42-fold difference between IC_{50}s of BW284c51 with recombinant mosquito and greenbug AChE is due to species differences, given that both insects express *ace*-1 orthologues (Gao et al. 2002; Huchard et al. 2006; Weill et al. 2002). Our data for BW284c51 is consistent with previous data that shows *bis* quaternary ligands exert their potency through the interaction with W84 (Harel et al. 1993), a conserved residue in the AChE phenotypes used in this study. The fairly low potency of BW284c51 to ChkAChE, compared to other AChEs may result from the lack of W279, Y121 and Y70, which have been shown to be crucial in their interaction with this compound (Eichler, 1994).

BW284c51 was least potent to ChkAChE, compared to the other AChEs, but was the most potent ligand to ChkAChE. The dual quaternary nature of BW284c51 apparently can compensate for the lack of PAS structural moieties in ChkAChE. The compromised integrity of the 'impaired' PAS of ChkAChE is further illustrated by the complete lack of potency with ethidium and propidium, both of which are AChE peripheral site ligands. Low potency of PRC 472 to Akron *Ag*AChE-G119S compared to WT *Ag*AChE-G3 suggests that the serine hydroxyl group can affect binding of PRC 472.

Propidium and ethidium are specific ligands of the peripheral site (Taylor and Lappi 1975; Taylor and Radic, 1994; Berman et al. 1987). Although propidium and ethidium are comparatively weaker inhibitors, their greater potency to both HuAChE and *Ag*AChE-I70Y further elucidates the structural relatedness between these 2 enzymes at

the peripheral anionic site. Comparative potency of propidium and ethidium is highly variable across AChEs, and reveals that ethidium is a better inhibitor than propidium. Data obtained in this study colloborates previous findings by Eichler et al. (1993), which showed no inhibition of ChkAChE at 1 mM of propidium, despite it being a sub-micromolar inhibitor of *Tc*AChE. In the same study, Eichler and co-workers showed superior affinity of Bw284c51, compared to propidium, and their observations agree with the findings of this study. I also note that propidium and ethidium are 12-19-fold selective for HuAChE than mosquito and Chicken AChEs. It remains to be investigated to what extent this selectivity is conserved across other vertebrate AChEs and to be compared with AChE-2 bearing insects such as *Drosophila*.

5.4.3 I70Y decreases substrate affinity in Ag*AChE but has no significant effect on catalytic efficiency*

Comparisons of *Ag*AChE-LiN and *Ag*AChE-I70Y substrate kinetics with ATChI revealed that substrate affinity decreases significantly in I70Y, but their maximal velocities (Vmax) are not significantly different. Interestingly, the Km values of both HuAChE and *Ag*AChE-I70Y with ATChI are not significantly different (P > 0.05; Table 5.5), thereby providing another diagnostic index of similarity between these two AChEs. Data further reveals that, while *Ag*AChE-LiN had the lowest Km value (highest affinity for ATChI), the increased substrate affinity again did not correspond to faster maximal reaction rates. Km value obtained for *Ag*AChE-LiN is 2-fold higher than that of a purified recombinant AChE of the greenbug, *Schizaphis graminum* (Zhao et al. 2010), indicating low variability in affinity for ATChI with these insect AChEs. Previous

150

studies report that insect AChEs have lower specific activity than vertebrate AChEs (Krupka and Hellenbrand, 1974; Gnagey, 1987; Toutant, 1989), hence further characterization of the enzymes used in the current study is required to explore this possibility.

In a recent related study, Jiang et al. (2009) reported that the Km values for a recombinant *Ag*AChE with ATChI was approximately 64 μM. Their value was 3-fold lower than what I obtained in the current study, with *Ag*AChE-LiN. Observed disparities in Km and Vmax values with ATChI may result from the fact that Jiang *et al.* (2009) assessed these parameters using purified enzyme, whereas in the current study, both *Ag*AChE-LiN and *Ag*AChE-I70Y were unpurified cell lysates. Since the primary focus of my objective was not to exhaustively characterize *Ag*AChE-LiN, a more conclusive comparison of enzyme substrate kinetics between Jiang's recombinant enzyme and *Ag*AChE-LiN, which both share the same phenotype, will be reported elsewhere. Detailed comparative enzyme kinetics of HuAChE, *Ag*AChE-LiN and *Ag*AChE-I70Y with several substrates as well as inhibition by a wider array of catalytic and peripheral site ligands will be addressed in the future.

References

Anderson TD, Paulson SL, Wong DM, Carlier PR, and Bloomquist JR (2008). Pharmacological mapping of the acetylcholinesterase catalytic gorge in mosquitoes with bis(n)-tacrines *In* Advances in Human Vector Control. *ACS Symposium Series*, 1014 (10): 143–151.

Berman HA, Decker MM, Nowak MW, Leonard KJ, McCauley M, Baker WM and Taylor P (1987). Site selectivity of fluorescent (bis)quaternary phenanthridinium ligands for acetylcholinesterase. *Mol Pharmacol*. 31: 610–616.

Cardozo MG, Kawai T, Imura Y, Sugimoto H, Yamanishi Y, and Hopfinger AJ (1992). Conformational analyses and molecular-shape comparisons of a series of indanone-benzylpiperidine inhibitors of acetylcholinesterase. *J. Med. Chem*. 35: 590-601.

Carlier PR, Anderson TD, Wong DM, Hsu DC, Hartsel J, Ma M, Wong EA, Choudhury R, Lam PCH, Totrov MM, and Bloomquist JR (2008). "Towards a species-selective acetylcholinesterase inhibitor to control the mosquito vector of malaria, *Anopheles gambiae*" *Chemico-Biol. Inter*. 175: 368-375.

Changeux JP (1966). Responses of acetylcholinesterase from *Torpedo marmorata* to salts and curarizing drugs. Mol Pharmacol. 2: 369-392.

Cheng, D.H., Ren, H. & Tang, X.C. (1996). Huperzine A, a novel promising acetylcholinesterase inhibitor. *Neuroreport* 8: 97-101.

Colletier JP, Fournier D, Greenblatt HM, Stojan J, Sussman JL, Zaccai G, Silman I, and Weik M (2006). Structural insights into substrate traffic and inhibition in acetylcholinesterase. *The EMBO J*. 25: 2746-2756.

Dougherty D (1996). Cation-pi interactions in chemistry and biology: A new view of benzene, Phe, Tyr, and Trp. *Science* 271: 163-168.

Durán R, Cerveñansky C, Dajas F, and Tipton KF (1994). Fasciculin inhibition of acetylcholinesterase is prevented by chemical modification of the enzyme at a peripheral site. *Biochim. biophys. Acta* 1201: 381-388.

Eichler J, Anselmet A, Sussman, Massoulié J, and Silman I (1994). Differential effect of "peripheral site" ligands on Torpedo and chicken acetylcholinesterase. *Molec. Pharmac*. 45: 395-398.

Ellman G, Courtney K, Andres V, Feather-Stone R (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol*. 7: 88-95.

Galli A, Mori F, Benini L, and Cacciarelli N (1994). Acetylcholinesterase protection and the anti-diisopropylfluorophosphate efficacy of E2020. *Eur. J. Pharmacol*. 270, 189-193.

Gao JR, Kambhampati S, Zhu KY (2002). Molecular cloning and characterization of a greenbug (Schizaphis graminum) cDNA encoding acetylcholinesterase possibly evolved from a duplicate gene lineage. *Insect Biochem. Mol. Biol*. 32: 765-775.

Gentry MK, and Doctor BP (1991). Alignment of amino acid sequences of acetylcholinesterase and butyrylcholinesterase, In *Cholinesterases: Structure Function Mechanism, Genetics and Cell biology* (B. P. Doctor and D.M Quinn et al. *eds*). ACS, Washington D.C. pp 394-398.

Gnagey AL, Forte M, and Rosenberry TL (1987). Isolation and characterization of acetylcholinesterase from Drosophila. *J. Biol. Chem*. 262: 13290-13298.

Harel M, Quinn DM, Nair HK, Silman I, and Sussman JL (1996). The X-ray structure of a transition state analog complex reveals the molecular origins of the catalytic power and substrate specificity of acetylcholinesterase. *J. Am. Chem. Soc*. 118, 2340-2346.

Harel M, Schalk I, Ehret-Sabatier L, Bouet F, Goeldner M, Hirth C, Axelsen PH, Silman I, and Sussman JL (1993). Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. *Proc. Natl Acad. Sci. USA* 90: 9031-9035.

Harel M, Sussman JL, Krejci E, Bon S, Chanal P, Massoulie J, and Silman I (1992). Conversion of acetylcholinesterase to butyrylcholinesterase: modeling and mutagenesis. *Proc. Natl. Acad. Sci. USA* 89: 10827-10831.

Haviv H, Wong DM, Silman I, Sussman JL. (2007) Bivalent ligands derived from Huperzine A as acetylcholinesterase inhibitors. *Curr. Top. Med Chem*. 7(4):375-87.

Huchard E, Martinez M, Alout H, Douzery EJ, Lutfalla G, Berthomieu A, Berticat C, Raymond M, Weill M (2006). Acetylcholinesterase genes within the Diptera: Takeover and loss in true flies. *Proc R Soc Lond, Ser B: Bio Sci* 273: 2595-2604.

Inoue A, Kawai T, Wakita M, Imura Y, Sugimoto H, and Kawakami, Y. (1996). The simulated binding of (+/–)-2,3-dihydro-5,6-dimethoxy- 2-[(1-(phenylmethyl)-4-piperidinyl)methyl]-1H-inden-1-one hydrochloride (E2020) and related inhibitors to free and acylated acetylcholinesterases and corresponding structure-activity analyses. *J. Med. Chem*. 39, 4460-4470.

Jiang A, Liu S, Zhao P and Pope C (2009). Recombinant expression and biochemical characterization of the catalytic domain of acetylcholinesterase-1 from the African malaria mosquito, *Anopheles gambiae*. *Insect Biochem. Mol. Biol*. 39(9): 646-53

Kawakami Y, Inoue A, Kawai T, Wakita M, Sugimoto H, and Hopfinger AJ (1996). The rationale for E2020 as a potent acetylcholinesterase inhibitor. *Bioorg. Med. Chem*. 4: 1429-1446.

Krupka RM, and Hellenbrand K (1974). Specific versus non-specific interactions of mammalian and insect acetylcholinesterase with substrates and reversible inhibitors. *Biochimica et Biophysica Acta (BBA) – Enzymology:* 370(1): 280-215.

Kryger G, Silman I, and Sussman JL (1999). Structure of acetylcholinesterase complexed with E2020 (Aricept®): implications for the design of new anti-Alzheimer drugs. *Structure* 7: 297-307.

Loewenstein Y, Gnatt A, Neville LF, and Soreq H (1993). Chimeric human cholinesterase. Identification of interaction sites responsible for recognition of acetyl- or butyrylcholinesterase-specific ligands. *J. Mol. Biol*. 234: 289-296.

Muñoz-Ruiz P, Rubio L, García-Palomero E, Dorronsoro I, del Monte-Millán M, Valenzuela R, Usán P, de Austria C, Bartolini M, Andrisano V, Bidon-Chanal A, Orozco M, Luque FJ, Medina M, Martínez A (2005). Design, synthesis, and biological evaluation of dual binding site acetylcholinesterase inhibitors: new disease-modifying agents for Alzheimer's disease. *J. Med. Chem.* 48(23): 7223-33.

Mutunga JM, Anderson TD, Wong DM, Carlier PR, and Bloomquist JR (2009). Inhibition of *Blattella germanica* acetylcholinesterase by bis(n)-tacrines: Prospects for the molecular design of a selective insecticide for a household pest." Household, Structural and Residential Pest Management, *ACS Symposium Series* 1015, p. 41-51.

Nightingale SL (1997). Donepezil approved for treatment of Alzheimer's disease. *JAMA* 277: 10.

Pang Y-P, Singh SK, Gao Y, Lassiter TL, Mishra RK, et al. (2009) Selective and irreversible inhibitors of aphid acetylcholinesterases: Steps toward human-safe insecticides. *PLoS ONE* 4(2): e4349. doi:10.1371/journal.pone.0004349.

Polsinelli GA, Singh SK, Mishra RK, Suranyi R, Ragsdale DW, Pang Y-P, and Brimijoin S (2010). Insect-specific irreversible inhibitors of acetylcholinesterase in pests including the bed bug, the eastern yellowjacket, German and American cockroaches, and the confused flour beetle. *Chem. Biol. Inter*. 187(1-3): 142-147.

Radic Z, Duran R, Vellon DC, Li Y, Cervensansky C, and Taylor P (1994). Site of fasciculin interaction with acetylcholinesterase. *J. Biol. Chem.* 269: 11233-11239.

Shafferman A, Velan B, Ordentlich A, Kronman C, Grosfeld H, Litner M, Flashner Y, Cohen S, Barak D, and Ariel N (1992). Substrate inhibition of acetylcholinesterase: residues affecting signal transduction from the surface to the catalytic center. *EMBO J.* 11: 3561-3568.

Sussman JL, Harel M, and Silman, I (1992). Three-dimensional structure of acetylcholinesterase, In *Multidisciplinary approaches to Cholinesterase functions* (A. Shafferman and B. Velan, *eds*) Plenum Press, NY 95-107.

Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L, and Silman I (1991). Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine binding protein. *Science* (Washington D.C.) 253: 872-879.

Szegletes T, Mallender WD, and Rosenberry TL (1998). Nonequilibrium analysis alters the mechanistic interpretation of inhibition of acetylcholinesterase by peripheral site ligands. *Biochemistry* 37: 4206-4216

Taylor P, and Lappi S (1975). Interaction of fluorescence probes with acetylcholinesterase. Site and specificity of propidium binding *Biochemistry* 14: 1989–1997.

Taylor P, and Radic Z (1994). The cholinesterases: from genes to proteins. *Ann. Rev. Pharmaco. Toxico.* 34: 281-320.

Thomsen T, and Kewitz H (1990). Selective inhibition of human acetylcholinesterase by galanthamine *in vitro* and *in vivo*. *Life Sci.* 46: 1553-1558.

Toutant JP (1989). Insect acetylcholinesterase: catalytic properties, tissue distribution and molecular forms. *Prog. Neurobiol.* 32: 423-446.

Vellom DC, Radic Z, Li Y, Pickering NA, Camp S, and Taylor P (1993). Amino acid residues controlling acetylcholinesterase and butyrylcholinesterase activity. *Biochemistry* 32: 12-17.

Verdonk ML, Boks GJ, Kooijman H, Kanters JA, and Kroon J (1993). Stereochemistry of charged nitrogen-aromatic interactions and its involvement in ligand-receptor binding. *J. Comput. Aided Mol. Des.* 7: 173-182.

Wang H, Carlier P. Ho W, Wu D, Lee N, Li C, Pang Y-P and Han Y (1999). Effects of bis(7)-tacrine, a novel anti-Alzheimer's agent, on rat brain AChE. *NeuroReport* 10: 789-793.

Weill M, Fort P, Berthomieu A, Dubois MP, Pasteur N, Raymond M (2002). A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non-homologous to the ace gene in Drosophila. 269(1504): 2007-16.

Wong DM, Greenblatt HM, Dvir H, Carlier PR, Han YF, Pang YP, Silman I, and Sussman JL (2003). Acetylcholinesterase complexed with bivalent ligands related to Huperzine A: experimental evidence for species-dependent protein-ligand complementarity. *J. Am. Chem. Soc.* 125(2): 363-73.

Zhao P, Zhu KY, and Jiang H (2010). Heterologous expression, purification, and biochemical characterization of a Greenbug (*Schizaphis graminum*) acetylcholinesterase encoded by a paralogous gene (ace-1). *J. Biochem. Mol. Tox* 24(1): 51-59

Chapter 6. Conclusions and future studies

This dissertation describes the pharmacological differences of the AChE gorge across vertebrate and insect species. The property of bivalency and its relevance to designing dual binding AChE inhibitors is revisited with a quest to explore a new class of insecticides that are safer and effective for control of mosquitoes. A detailed account of inhibition and toxicological profiles of *N*-methylcarbamates and related analogs is also presented. I have dissected the role of metabolic detoxication and cuticular penetration in toxicity. New reports of candidate monovalent and bivalent carbamates that can be used for mitigating insecticide resistance were presented, as well as validation of a molecular model for selectivity of select PSL, using *in vitro* structural and functional enzyme assays.

The design of bivalent insecticide relies on the architecture of the AChE gorge, and especially the differences between target and non-target organisms. The highly conserved nature of AChE, both is structure and function calls for a fine-scale examination of AChE gorge geometry. While molecular modeling and sequence alignments provide insights to the amino acid composition of the AChE gorge, elucidating the interactions of amino acid side chains with bivalent ligands is important in correlating structure and function. Results presented in chapter 2 explored the differences between *Drosophila* and cockroach AChE, sensitivity to *bis*(n)-tacrines and compares current findings with other published literature. I conclude that stringent tether lengths are required and vary between 6 carbon to 10 carbon methylene linkers. More so, vertebrate and *Drosophila* AChE display a high tether length dependent potency enhancement, unlike mosquito and cockroach AChE, a trend that partitions similarly with

157

their AChE protein sequence alignments. Though non-insecticidal, tacrine dimers are important molecular rulers, which provide incisive and important pharmacological information on AChEs. I recommend future assays with groups of AChE-1 and AChE-2 bearing insects, to further dissect the AChE gorge geometrical differences between the two AChE phenotypes. Such data will be valuable in the molecular design of selective and effective bivalent insecticide, with an understanding of the target pest or disease vector population.

The emerging trend of increasing pyrethroid resistance in mosquito populations calls for an immediate need to have alternative insecticide to avert eminent failures in current malaria mosquito control programmes. Previous semi-field testing of pyrethroid-carbamate mixture or in rotations shows a promising strategy to control pyrethroid-resistant mosquito populations. Results presented and discussed in chapter 3 details the testing of new carbamate molecules that have greater mosquito selectivity than conventional methylcarbamates, yet comparatively toxic to mosquitoes. Despite the marginal mosquito toxicity in some of our most selective compounds compared to propoxur (the WHO standard), pharmacokinetic studies reveal key factors, such as cuticular penetration and metabolic detoxification; that can be targeted to scale-up toxicity to mosquitoes in redesigning or at the formulation stage of insecticide development. Metabolic detoxication was mainly mediated by P450-monooxygenases. A 3-substituted alkyl silyl compound (PRC 387) was not synergized by DEF, yet synergism in 2-alkyl substituted analogs was significant. I recommend future DEF synergism experiments with more side chain branched 3-alkyl silyl compounds to explore possibilities that esterases do not metabolize this class of compounds. Sulfur-containing

158

compounds did not show increased toxicity when injected, compared to topical applications, an observation that requires further studies of penetration factors. Future electrophysiological studies using intact and transected insect CNS should be done to investigate the ability of sulfur-containing compounds to penetrate the blood-brain barrier.

The effect of structural variation on selectivity showed that 2-substituted phenyl methylcarbamates have greater mosquito selectivity than their 3-substituted analogs. Optimal selectivity was observed with 2-substituted and not the 3-substituted phenyl *N*-methylcarbamates bearing alkyl O- or S-ethers. Optimal toxicity was observed at *sec*-butyl substituted analogs of phenyl *N*-methylcarbamates. Optimal structural configurations of carbamates tested re-enforce the importance of their structural and functional similarity to the substrate, ACh and the catalytic active site geometry. Selectivity factor was used in this study to predict safety in humans, but future toxicity testing in animal models is required to ascertain the postulated low human toxicity as a step to further development of these chemicals. Additionally, future studies are required to explain the underlying biophysical or neuronal mechanism of observed carbamate-induced leg loss in mosquitoes and quantify the direct impact to disease transmission in semi-field and field settings.

Chapter 3 also details the first report of trifluorinated actophenone carbamates and *N*-methylpyrazole carbamates that are toxic to both susceptible carbamate-resistant mosquitoes. These carbamates are good leads for not only generating safer carbamates, but also those that can be used in insecticide resistant mosquito populations. I also report the first potent and mosquito toxic bivalent carbamates, a subclass that establishes the

159

new paradigm of a bivalent strategy in combating resistance development and increasing selectivity to mosquitoes. In the future, a more complete series of bivalent compounds will be evaluated to exploit different combination chemistries of peripheral and catalytic site ligands. Although *N*-sulfenylated carbamates lacked contact toxicity, they are highly toxic when topically applied, this warrant further investigations. Future microinjection and synergism studies are required to help understand the cuticular penetration and metabolic fate of these compounds. Since metabolic de-fluorination is unlikely *in vivo*, the observed toxicity of PRC 515, a compound having low anticholinesterase activity, *in vitro*, cannot be accounted for, and therefore other underlying mechanisms of toxicity may come in play. I recommend future detailed toxicokinetic assays with PRC 515 and its analogs, enzyme-ligand docking and electrophysiological studies to further dissect its mechanism of toxicity to mosquitoes.

Potent and bivalent pyrazole-indanone N-methyl carbamates described in chapter 3 provide a promising chemistry, but their selectivity is yet to be evaluated. Piperidine derived PSLs were described in chapter 4 where by, attempts to scale up for toxicity and selectivity of PRC 472 were futile. Enzyme ligand docking studies should be done to further understand the mechanism of low potency of the piperidine analogs. Since piperidine derivatives were not toxic in mosquitoes even when injected, there is need for electrophysiological examination of their activity on insect CNS. This will pave the way for future synthesis and testing of piperidine-derived peripheral site ligands, supposedly having the great selectivity observed with PRC 472, and high mosquito toxicity conferred by a catalytic site ligand.

Effects of side chain branching and halogenation are detailed in chapter 4. Alkyl and silyl branching did not have different selectivity to mosquitoes although the former class was consistently more toxic to mosquitoes. Varying levels of improved selectivity was achieved in this study, but efforts to increase mosquito toxicity of the modified analogs were unsuccessful. From the various alkyl modifications studied we conclude that structural complementarity to the substrate ACh is crucial to maintaining potency. Although potency and selectivity of branched thioalkyl carbamates increases with alkyl chain length, their toxicity is compromised beyond C_3. Future exploration of mosquito-selectivity with thioalkyl-substituted compounds should not exceed C_3.

An ethyl-S-phenyl substituted *N*-methylcarbamate (PRC 529) was potent to *Ag*ACHE, yet inactive with HuACHE, thereby resulting to the highest selectivity recorded in this study (S > 412). Since this compound was toxic to mosquitoes, I recommend future topical assays, synergism and microinjection studies to further explore the toxicity of this compound. The anticipated bioactivation of thioalkyl compounds and halogen-mediated protection of metabolic detoxication did not occur *in vivo*, the reason for this is unclear and warrants further investigation. Di-substitution of alkyl side chains with halogens or thioalkyl moieties was unfavorable. The spatial and structural complementarity of the ligand to the substrate and the active site catalytic molecular signature is also of great importance for effective inhibition of the enzyme.

Lastly, chapter 5 explored the molecular mechanism of observed *in vitro* selectivity of PRC 472 to mosquito enzyme, by use of assays to examine the gorge geometry, inhibition potency by PSLs, and kinetic properties of WT and mutant AChEs. Structural and functional similarities between HuAChE and AgAChE-I70Y have been

clearly elucidated by the three indices used in this study. It is therefore true that the *Ag*-selectivity model for PRC 472 is well supported by these relevant *in vitro* functional studies. The discovery of PRC 472 is a great resource in designing ligands that explore the pharmacodynamic properties of bivalent carbamates that confers enhanced toxicity to mosquitoes *in vivo*. I recommend that molecular models and possibly crystal *Ag*AChE structures be generated to enable further exploration of the functional role of amino-acid moieties at the peripheral site and possibly inspire the design of new peripheral site ligands. The potency of peripheral site ligands should also be assessed based on fluorescent ligand binding assays with thioflavin-T (Sultatos and Kaushik, 2008) in order to understand the role of binding affinity and non-covalent modification of the substrate-binding site in inhibition. Data from such studies may help improve binding affinity of PSLs. A detailed characterization of kinetic properties of highly purified *Ag*AChE-I70Y, *Ag*AChE0LiN, *Ag*AChE-G119S and ChkAChE should be performed to better understand in detail the effects of various mutations on substrate affinity and catalytic efficiency. Such studies will enable the comparison of the data collected with other relevant published results.

References

Sultatos L and Kaushik R (2008). Altered binding of thioflavin t to the peripheral anionic site of acetylcholinesterase after phosphorylation of the active site by chlorpyrifos oxon or dichlorvos. *Toxicol. Appl. Pharmacol.* 230(3): 390-6.

Appendix A

164

Appendix B

Show this HTML in a new window?

3-Mar-2011 1:10am Rehovot, ISRAEL
Dear James
You have my permission to use the figures from our 2006 paper:
E.H. Rydberg, B. Brumshtein, H.M. Greenblatt, D.M. Wong, D. Shaya, L.D. Williams, P.R. Carlier, Y.-P. Pang, I. Silman, J.L. Sussman, Complexes of alkylene-linked tacrine dimers with Torpedo californica acetylcholinesterase: Binding of bis(5)-tacrine produces a dramatic rearrangement in the active-site gorge, J. Med. Chem. 49 (2006) 5491-5500.
With best regards,
Joel

--
Prof. Joel L. Sussman, Pickman Professor of Structural Biology
Director, The Israel Structural Proteomics Center
Dept. of Structural Biology
Weizmann Institute of Science
Rehovot 76100 ISRAEL
Tel: +972 8-934 4531; Fax: +972 8-934 6312
E-mail: Joel.Sussman@weizmann.ac.il
Web: www.weizmann.ac.il/~joel www.weizmann.ac.il/ISPC
proteopedia.org

--

On 3 Mar 2011, at 00:11, James Mutunga wrote:

Dear Dr. Sussman

I would like to use some of the figures in your 2006 paper, see:
(http://www.weizmann.ac.il/ISPC/papers/2006_Rydberg_JMedChem.pdf).
I am a PhD student at Virginia Tech, working with Dr. Dawn Wong who is a co-author of the paper. I would use the figures in my dissertation, and here by request for permission.