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

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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

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April 28, 2011
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Key words: *N*-methylcarbamates, active site gorge, tacrine dimers, selectivity, Acetylcholinesterase, oximes, pyrazoles, Bivalent carbamates, Akron strain

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**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 carboxamates 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

I would like to sincerely thank my advisor Dr. Jeffrey R. Bloomquist for offering me the opportunity to pursue my doctoral studies. He also provided exemplary guidance, encouragement and support during my studies; it was a great honor to have worked with him. I also express my heartfelt gratitude to my advisory committee Dr. Kevin Myles, Dr. Igor Sharakhov, Dr. Sally Paulson and Dr. Troy Anderson for the support, guidance and great advice. I thank Dr. Paul Carlier and his team for providing the experimental carbamates used in this study. I extend my gratitude to Dr. Carlier’s postdoctoral scientist namely, Dr. Wong, Dr. Ma, Dr. Craft and Dr. Chen and graduate students Joshua Hartsel and Astha Astha. I also thank Dr. Li for providing the recombinant mutant enzymes and Dr. Marion Ehrich for providing chicken enzyme. I am indebted to Dr. Polo Lam and Dr. Max Totrov, both of Molsoft LLC for the molecular modeling work and advice.

I thank Dr. John Githure, James Wauna and other members of malaria research program at the International Center of Insect Physiology and Ecology (ICIPE) Kenya, for their help with field sampling of mosquitoes. I am highly indebted to my colleagues at the department namely; Brenna Traver, Gina Davis, Lacey Jenson and Daniel Swale for their assistance and continued support. The Sabatia family ensured a homely environment far from home and I take this chance to sincerely them. I am indebted to Dr. Judy Mollet for her continued support and encouragement. Lastly I acknowledge my dear wife Mercy Kathambi, our son Allan Mutunga and daughter Shanese Mwende, and our parents for their unconditional support, patience and prayers. I thank the NIH and IVCC for funding and give thanks to God for the gift of life and good health.
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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 \textit{ace}-1 gene in mosquitoes) and metabolic resistance (Brogdon and McAllister, 1998). Target site insensitivity has been reviewed extensively in insects (Ranson \textit{et al.}, 2004; Brogdon and McAllister, 1998; Fournier and Mutero, 1994). Target site insensitivity in insects is associated with several different mutations in \textit{ace}-1 and \textit{ace}-2 genes, which code for AChE-1 and AChE-2, respectively. Some insects have both genes but only the \textit{ace}-1 is functionally important in neurotransmission (Bourguet \textit{et al.}, 1996; Weill \textit{et al.}, 2003). Mutations involving \textit{ace}-2 have only been reported in three dipteran species namely, \textit{Drosophila melanogaster}, \textit{Musca domestica} and \textit{Bactrocera oleae} (Mutero \textit{et al.}, 1994; Menozzi \textit{et al.}, 2004; Kozaki \textit{et al.}, 2001; Vontas \textit{et al.}, 2002). Point mutations in the \textit{ace}-2 gene have been described in insecticide resistant strains of \textit{D. melanogaster} (Mutero \textit{et al.}, 1994; Fournier \textit{et al.}, 1992). Mutations in the \textit{ace}-2 gene of \textit{M. domestica} and \textit{B. oleae} have been described (Walsh \textit{et al.}, 2001; Vontas \textit{et al.}, 2002). Studies in Central American and Mexican populations of \textit{An. albimanus} have shown that an altered AChE is the most common OP/ CB resistance mechanism (Penilla \textit{et al.}, 1998, Ayad and Georghiou, 1979; Hemingway and Georghiou, 1983). AChE target-site insensitivity has been reported from \textit{Culex pipiens}, \textit{Cx. quinquefasciatus}, \textit{Cx. tritaeniorhynchus}, \textit{An. nigerimus}, \textit{An. atroparvus}, \textit{An. sacharovi} and \textit{An. gambiae} (Bisset \textit{et al.}, 1990; N’Guessan \textit{et al.}, 2003). A G119S mutation in the \textit{ace}-1 gene has been identified in target site insensitive AChE strains of \textit{Cx. pipiens}, \textit{Cx. quinquefasciatus} and \textit{An. gambiae} (Weill \textit{et al.}, 2002; Weill \textit{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 (Djogbénon 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) conferring 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 phosphorothionatate 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 \textit{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 \textit{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 \textit{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 \textit{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.
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 (0.1-0.4 g/m²) and propoxur WP (1-2 g/m²) 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 LD₅₀ values in rats (Minton and Murray, 1988). Class I is most toxic (e.g. chlorfenvinphos) and has an LD₅₀ in the range 1-30 mg/kg. The oral LD₅₀ 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 (2 g/m²), malathion WP (2 g/m²) and pirimiphos-methyl WP (1-2 g/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 into 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 AgAChE peripheral site is absent in humans (Pang, 2006). Homology models have also shown differences near the peripheral and active sites of AgAChE and hAChE, notably I70/Y72, A81/T83, Y333/G342, M348/P446, D441/Y449, (AgAChE/hAChE 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;
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
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).
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).

![Docking of a bivalent tacrine anticholinesterase (7 carbon tether) with TcAChE](Reproduced with permission; Rydberg et al., 2006).

**Figure 1.4.** Docking of a bivalent tacrine anticholinesterase (7 carbon tether) with TcAChE (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 AgAChE. 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.


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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 melanogaster, which express AChE-1 and AChE-2 isoforms, respectively. Overall potency of bis(n)-tacrines was greater in D. melanogaster AChE (DmAChE) than in B. germanica AChE (BgAChE). Change in potency with tether length was high in DmAChE and low in BgAChE, 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\textsubscript{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)-tacrine (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.

![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.](image)

**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°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)-tacrine 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)-tacrine 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 TcAChE, BgAChE, and DmAChE 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 TcAChE (PDB ID 2ACE) (Raves et al. 1997; Millard et al. 1999) and DmAChE (PDB ID 1dx4) (Harel et al. 2000). The alignment of the flexible peripheral site loop, around the deletion region for BgAChE and DmAChE 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 BgAChE (Q1-N544) was numbered based on the mature form of TcAChE, beginning at residue Q109 from the full-length sequence, Q2PZG3_BLAGE (Bg ace-1). Unless otherwise stated, sequence identities and similarities to BgAChE were calculated using the BLAST 2 sequence program (bl2sq) from NCBI, with default settings (Tatusova and Madden, 1999), and based on alignments of hAChE, mAChE, and DmAChE against Q1-N544 of BgAChE, which corresponds to the mature catalytic subunit in TcAChE.

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 BgAChE (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 TcAChE (PDB ID 1W4L, 2.16 Å) (Greenblatt
et al. 2004), mAChE (PDB ID 1N5R, 2.25 Å; chain A) (Bourne et al. 2003), and E202Q mutant hAChE (PDB ID 1F8U, 2.90 Å) (Kryger et al. 2000). The hAChE template was chosen by the best-fit option of Modeller (6v2), which shares 35.9% sequence identity with BgAChE (using the ALIGN program therein). By contrast, the TcAChE and mAChE templates used for homology modeling were chosen owing to their high resolution structural data, which share 35.3% and 34.6% sequence identity with BgAChE, 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 BgAChE. 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 bis(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. 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 \textit{bis}(8)-tacrine. In contrast, the plot for \textit{Drosophila} shows declining IC$_{50}$s with increasing tether length, with bumps at \textit{bis}(2)- and \textit{bis}(4)-tacrine, along with a general plateau occurring from \textit{bis}(5)-tacrine to \textit{bis}(12)-tacrine (Fig. 2.3). Within the plateau region, the differences in \textit{DmAChE} 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 \textit{bis}(n)-tacrine (numerator) versus that of tacrine monomer (denominator). The most potent tacrine dimer to \textit{BgAChE} and \textit{DmAChE} were \textit{bis}(8)- and \textit{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 \textit{DmAChE} present 1.7-fold and 2-fold decreases in potency, with the highest potency loss of 152-fold occurring between \textit{bis}(2)- and \textit{bis}(10)-tacrine, compared to only 27-fold in \textit{BgAChE}. \textit{DmAChE} had only \textit{bis}(2)-tacrine with a TLDI value <1, meaning that potency was lower than with monomer. In contrast, there are four such values in \textit{BgAChE} namely; \textit{bis}(2)-, (3)-, (4)- and (12)-tacrine.

Following the observed overall enhanced potency to \textit{DmAChE} compared to \textit{BgAChE}, I computed the potency ratio (PR: \textit{Bg} IC$_{50}$/\textit{Dm} IC$_{50}$) for each tacrine dimer (Table 2.1). Enhanced \textit{DmAChE} potency compared to \textit{BgAChE} increased with tether length, and the difference is maximal at \textit{bis}(12)-tacrine (PR= 297). At \textit{bis}(10)-tacrine, potency to \textit{DmAChE} is 137-fold higher than in \textit{BgAChE}; 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.

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

*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 BgAChE and DmAChE, respectively. Potency ratio (PR) = (IC$_{50}$ with BgAChE)/(IC$_{50}$ with DmAChE) indicating the selectivity in potency of D. melanogaster by dimeric tacrines compared to B. germanica.
Figure 2.3. Plots of AChE IC\(_{50}\) (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 BgAChE compared to weak pi-pi interactions of the Y334 in DmAChE, which may allow for more flexibility of the enzyme. This flexibility at the PAS may explain the low change in DmAChE 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 (BgAChE numbering).

<table>
<thead>
<tr>
<th></th>
<th>catalytic triad</th>
<th>oxyanion hole</th>
<th>choline-binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tc</td>
<td>TIFGESAGGAS</td>
<td>E H</td>
<td>YGGF</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>327</td>
<td>116 201</td>
</tr>
<tr>
<td>Bg ace-1</td>
<td>TLFGESECAVS</td>
<td>E H</td>
<td>YGGF</td>
</tr>
<tr>
<td></td>
<td>292</td>
<td>328</td>
<td>119 203</td>
</tr>
<tr>
<td>Dm ace-2</td>
<td>TLFGESEAGSSS</td>
<td>E H</td>
<td>YGGF</td>
</tr>
<tr>
<td></td>
<td>778</td>
<td>467</td>
<td>148 279</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>acyl pocket</th>
<th>peripheral site</th>
<th>peripheral site loop&lt;sup&gt;b&lt;/sup&gt; &lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tc</td>
<td>W F</td>
<td>Y D Y W YG</td>
</tr>
<tr>
<td></td>
<td>799</td>
<td>799 779 796 779 796</td>
</tr>
<tr>
<td>Bg ace-1</td>
<td>W C</td>
<td>I D Y W YY</td>
</tr>
<tr>
<td></td>
<td>255</td>
<td>255 258 251 258 255</td>
</tr>
<tr>
<td>Dm ace-2</td>
<td>W L</td>
<td>E Y M W YD</td>
</tr>
<tr>
<td></td>
<td>271</td>
<td>271 328 330 328 330</td>
</tr>
</tbody>
</table>

**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).<sup>a</sup>The peripheral site loop region has previously been referred to as the “acyl pocket loop” (Radic and Taylor, 2006) and in TcAChE 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.

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 C2-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 BgAChE 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\textsubscript{2} bump seems to be conserved in both AChEs, and is joined by a C\textsubscript{4} bump in \textit{DmAChE}. Longer tethers in \textit{DmAChE} present a different scenario not observed with \textit{BgAChE}. Notably, \textit{bis(12)}-tacrine is the least potent in \textit{BgAChE}, compared to \textit{bis(2)}-tacrine in \textit{DmAChE}. Moreover, \textit{bis(12)}-tacrine is ca. 4-fold less potent compared to most potent compound for \textit{DmAChE}, whereas, it is 27-fold less potent in \textit{BgAChE}. Additionally, the most potent ligand is 2 carbons longer in \textit{DmAChE} than in \textit{BgAChE}, and there is no drastic loss in potency at longer tethers in \textit{DmAChE}, as observed with \textit{BgAChE}. These observations suggest possible key structural moieties that cause differential ligand-enzyme interactions in the two species. It is unclear if \textit{bis(12)}-tacrine spanned the entire length of the \textit{DmAChE} gorge (CAS to PAS), since no major loss in potency was observed with this species. Screening \textit{DmAChE} 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 \textit{DmAChE} than in \textit{BgAChE} (Table 2.1). This means that change of tether lengths led to higher potency change in \textit{DmAChE} than \textit{BgAChE}, 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 \textit{DmAChE}, compared to \textit{BgAChE}. 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 *DmAChE* is not an ideal model for assessing selectivity of carbamate insecticides to insect pests and disease vectors.
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 neostigmine, 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 BgAChE and DmAChE. 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


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, \textit{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 TcAChE (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-monoxygenases 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
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 \((\text{ace-1}^R)\), which confers resistance to carbamates. Mosquito colonies are maintained at the Fralin Life Science Center insectary, at 75\% relative humidity and 25 °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:**

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

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).
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.
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\textsubscript{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 \( \mu \)L 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\textsubscript{50}). I used the ratio of LD\textsubscript{50} and ID\textsubscript{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 \textit{et al.} 2008), but \textit{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 \textit{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.

![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.](image)

**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 °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’ ACGATMACGTTCYTCCGA 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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Contact toxicity LC$\text{50}$, µg/mL</th>
<th>Topical toxicity, LD$\text{50}$, ng/insect</th>
<th>Injected toxicity, ID$\text{50}$, ng/insect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No synergist</td>
<td>+PBO</td>
<td>+DEF</td>
</tr>
<tr>
<td>Propoxur</td>
<td>37 (27-55)$^a$</td>
<td>2.1 (1.5-2.7)$^{a,1}$</td>
<td>1.8 (1.4-2.4)$^{a,1}$</td>
</tr>
<tr>
<td>PRC 331</td>
<td>31 (14-60)$^a$</td>
<td>1.7 (1.4-2.0)$^{a,2}$</td>
<td>1.8 (1.4-2.6)$^{a,b,2}$</td>
</tr>
<tr>
<td>PRC 337</td>
<td>69 (53-88)$^b$</td>
<td>3.4 (2.6-4.2)$^{b,2}$</td>
<td>3.4 (2.5-4.4)$^{b,2}$</td>
</tr>
<tr>
<td>PRC 387</td>
<td>169 (162-176)$^c$</td>
<td>2.1 (1.2-3)$^{a,b,2}$</td>
<td>6.7 (5-8.5)$^{c,1}$</td>
</tr>
<tr>
<td>PRC 407</td>
<td>1000 (70%)</td>
<td>2.7 (2-3.5)$^{a,b,2}$</td>
<td>3.4 (2.4-4.4)$^{b,2}$</td>
</tr>
<tr>
<td>PRC 408</td>
<td>1000 (27%)</td>
<td>2.7 (1.8-3.6)$^{a,b,2}$</td>
<td>3.3 (2.6-4)$^{b,2}$</td>
</tr>
<tr>
<td>PRC 421</td>
<td>1000 (27%)</td>
<td>18 (16-20)$^{c,2}$</td>
<td>38 (33-43)$^{d,3}$</td>
</tr>
</tbody>
</table>

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.
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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>CoSR</th>
<th>PtSR</th>
<th>SDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propoxur</td>
<td>1.5</td>
<td>2.3</td>
<td>1.5</td>
</tr>
<tr>
<td>PRC 331</td>
<td>2.6</td>
<td>8</td>
<td>3.1</td>
</tr>
<tr>
<td>PRC 337</td>
<td>3.6</td>
<td>4</td>
<td>1.1</td>
</tr>
<tr>
<td>PRC 387</td>
<td>3.8</td>
<td>3.3</td>
<td>0.87</td>
</tr>
<tr>
<td>PRC 407</td>
<td>4.0</td>
<td>3.8</td>
<td>0.95</td>
</tr>
<tr>
<td>PRC 408</td>
<td>3.8</td>
<td>2.5</td>
<td>0.66</td>
</tr>
<tr>
<td>PRC 421</td>
<td>4.4</td>
<td>6.2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

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,
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.

<table>
<thead>
<tr>
<th>Carbamate</th>
<th>$^{a}$G3 LC$<em>{50}$ or LD$</em>{50}$</th>
<th>$^{a}$Akron LC$<em>{50}$ or LD$</em>{50}$</th>
<th>(RR)$^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propoxur</td>
<td>37 $\mu$g/mL</td>
<td>0% @ 5000 $\mu$g/mL</td>
<td>&gt;135</td>
</tr>
<tr>
<td>Aldicarb</td>
<td>70 $\mu$g/mL</td>
<td>33 $\mu$g/mL</td>
<td>0.5</td>
</tr>
<tr>
<td>Methomyl</td>
<td>24 $\mu$g/mL</td>
<td>0% @ 5000 $\mu$g/mL</td>
<td>-</td>
</tr>
<tr>
<td>Bendiocarb</td>
<td>15 $\mu$g/mL</td>
<td>0% @ 5000 $\mu$g/mL</td>
<td>-</td>
</tr>
<tr>
<td>PRC 331</td>
<td>4.5 ng</td>
<td>348 ng</td>
<td>77</td>
</tr>
<tr>
<td>PRC 521</td>
<td>1.6 ng</td>
<td>27 ng</td>
<td>17</td>
</tr>
<tr>
<td>PRC 608</td>
<td>41 $\mu$g/mL</td>
<td>716 $\mu$g/mL</td>
<td>17</td>
</tr>
<tr>
<td>PRC 431</td>
<td>290 $\mu$g/mL</td>
<td>0% @ 1000 $\mu$g/mL</td>
<td>-</td>
</tr>
</tbody>
</table>

$^{a}$LC$_{50}$ values are $\mu$g/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.

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

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 unsubstituted 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 alphanates, \(N\), \(N\)-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.

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

aToxicity 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. bRR 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 cyclopentyl 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.

![Chemical structures of sulfenylated analogs of PRC 331 and 387.](image)

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<sub>50</sub> = 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 \textit{An. gambiae}.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Contact Toxicity\textsuperscript{a}</th>
<th>Topical Toxicity\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC 654</td>
<td>0% @ 1 mg/mL</td>
<td>NT</td>
</tr>
<tr>
<td>PRC 655*</td>
<td>70% @ 0.5 mg/mL</td>
<td>2.0 ng (1.8-2.3)</td>
</tr>
<tr>
<td>PRC 656</td>
<td>0% @ 1 mg/mL</td>
<td>NT</td>
</tr>
<tr>
<td>PRC 657</td>
<td>100% @ 1 mg/mL</td>
<td>ND</td>
</tr>
<tr>
<td>PRC 658</td>
<td>0% @ 1 mg/mL</td>
<td>NT</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Toxicity at 24 hrs determined by WHO treated filter paper assay and \textsuperscript{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\textsubscript{50} = 63 ng (55-72) and Akron LD\textsubscript{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

**Figure 3.11.** Chemical structures of experimental bivalent carbamates
Table 3.7. Toxicity of bivalent carbamates to G3 mosquitoes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>G3 Contact Toxicity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Compound</th>
<th>G3 Contact Toxicity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC 590</td>
<td>16% @ 1 mg/mL</td>
<td>PRC 603</td>
<td>0% @ 1 mg/mL</td>
</tr>
<tr>
<td>PRC 591</td>
<td>28% @ 1 mg/mL</td>
<td>PRC 624</td>
<td>20% @ 1 mg/mL</td>
</tr>
<tr>
<td>PRC 592</td>
<td>12% @ 1 mg/mL</td>
<td>PRC 625</td>
<td>0% @ 1 mg/mL</td>
</tr>
<tr>
<td>PRC 599</td>
<td>0% @ 1 mg/mL</td>
<td>PRC 646</td>
<td>10% @ 1 mg/mL</td>
</tr>
<tr>
<td>PRC 600</td>
<td>0% @ 1 mg/mL</td>
<td>PRC 647</td>
<td>10% @ 1 mg/mL</td>
</tr>
<tr>
<td>PRC 601</td>
<td>0% @ 1 mg/mL</td>
<td>PRC 648</td>
<td>0% @ 1 mg/mL</td>
</tr>
<tr>
<td>PRC 602</td>
<td>0% @ 1 mg/mL</td>
<td>PRC 649</td>
<td>0% @ 1 mg/mL</td>
</tr>
</tbody>
</table>

<sup>a</sup>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-buty1phenylmethylcarbamate) 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-monoxygenases 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 pattern 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
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 \textit{in vivo}. This is a likely scenario considering the fact that Akron mosquitoes possess both ace-1 and pyrethroid (\textit{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.

\subsection*{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 substituent (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 propesicidal. 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 LC₅₀ = 31 µg/mL) and 387 (G3 LC₅₀ = 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 (LD₅₀ = 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 alloxanate (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


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 Me₃N⁺ 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 AgAChE.

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.

\[
\text{Selectivity (S)} = \frac{A_g \text{AChE } IC_{50}}{Hu \text{AChE } IC_{50}}
\]

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.

<table>
<thead>
<tr>
<th>Cmpd R</th>
<th>AgAChE IC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
<th>HuAChE IC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
<th>S</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;, µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC 331 tert-butyl</td>
<td>36 (34-38)&lt;sup&gt;a,1&lt;/sup&gt;</td>
<td>320 (293-349)&lt;sup&gt;a,2&lt;/sup&gt;</td>
<td>9</td>
<td>31 (14-60)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 521 sec-butyl</td>
<td>17 (15-19)&lt;sup&gt;b,1&lt;/sup&gt;</td>
<td>25 (20-31)&lt;sup&gt;b,1&lt;/sup&gt;</td>
<td>1.5</td>
<td>31 (29-33)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 540 tert-pentyl</td>
<td>35 (33-36)&lt;sup&gt;a,1&lt;/sup&gt;</td>
<td>48 (37-60)&lt;sup&gt;c,2&lt;/sup&gt;</td>
<td>1.4</td>
<td>68 (64-72)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 550 2-Me-pentan-2-yl</td>
<td>11 (7-18)&lt;sup&gt;b,1&lt;/sup&gt;</td>
<td>12 (6-24)&lt;sup&gt;b,1&lt;/sup&gt;</td>
<td>1.1</td>
<td>236 (210-259)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 563 3-Me-pentan-3-yl</td>
<td>47 (33-66)&lt;sup&gt;a,1&lt;/sup&gt;</td>
<td>42 (22-82)&lt;sup&gt;c,1&lt;/sup&gt;</td>
<td>0.9</td>
<td>228 (214-228)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*Values in brackets are 95% CI. S = Mosquito selectivity = HuAChE IC<sub>50</sub>/AgAChE IC<sub>50</sub>. IC<sub>50</sub>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.</sup>

Substitution of the tert-butyl with a sec-butyl (PRC 331 to PRC 521) led to a ca. 2-fold gain in AgAChE 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 AgAChE, 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.
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. 2. Chemical structures of PRC 331 and its analogs bearing phenyl substituted thioalkyl groups.

Figure 4. 3. Metabolic bioactivation pathway of thioalkyl groups to more toxic sulfoxide and sulfone derivatives, \textit{in vivo}.
Addition of a Me-S- group at a position \textit{para} to the tert-butyl group (PRC 564) caused a drastic 27-fold loss in potency to \textit{AgAChE}, compared to PRC 331, the reference compound (Table 4.2). This also caused a complete loss of toxicity to mosquitoes at 1000 \(\mu\text{g/mL}\), our diagnostic maximum dose. Interestingly, this modification led to a mere 2-fold loss in potency to \textit{HuAChE}. 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 \textit{AgAChE}, when compared to PRC 564 (methyl-thio) and PRC 331, respectively. In \textit{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 \textit{iso}-propyl, but still retained the \textit{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 \textit{AgAChE}, and \textit{HuAChE}, respectively (Table 4.2).
Table 4.2. Potency and toxicity of thioalkyl N-methylcarbamates to *An. gambiae* mosquitoes

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>$\text{A}<em>{g}\text{AChE-IC}</em>{50}^*, \text{nM}$</th>
<th>$\text{HuAChE-IC}_{50}^*, \text{nM}$</th>
<th>S</th>
<th>$\text{LC}_{50}^*$; $\mu\text{g/mL}$ or % mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC 564</td>
<td>$t$-butyl</td>
<td>Me-S</td>
<td>974 (830-1172)$^a,1$</td>
<td>641 (572-719)$^{a,2}$</td>
<td>0.66</td>
<td>0% @ 1mg/mL</td>
</tr>
<tr>
<td>PRC 556</td>
<td>$t$-butyl</td>
<td>$i$-Propyl-S</td>
<td>32210 (23060-63300)$^{b,1}$</td>
<td>4674 (3777-5784)$^{b,1}$</td>
<td>0.12</td>
<td>4% @ 1mg/mL</td>
</tr>
<tr>
<td>PRC 555</td>
<td>$i$-propyl</td>
<td>$i$-Propyl-S</td>
<td>75440 (N/A)</td>
<td>2205 (1653-2941)$^c$</td>
<td>0.03</td>
<td>16% @ 1mg/mL</td>
</tr>
<tr>
<td>PRC 529</td>
<td>H</td>
<td>Et-S</td>
<td>243 (216-276)$^c$</td>
<td>&gt;100,000 (N/A)</td>
<td>&gt;412</td>
<td>296 (279-314)$^a$</td>
</tr>
<tr>
<td>PRC 530</td>
<td>H</td>
<td>Propyl-S</td>
<td>114 (135-152)$^{d,1}$</td>
<td>2290 (2019-2596)$^{c,2}$</td>
<td>16</td>
<td>339 (321-361)$^b$</td>
</tr>
<tr>
<td>PRC 337</td>
<td>H</td>
<td>2-Me-allyl-S</td>
<td>77 (59-102)$^{e,1}$</td>
<td>5778 (5439-6123)$^{b,2}$</td>
<td>75</td>
<td>69 (53-58)$^c$</td>
</tr>
<tr>
<td>PRC 407</td>
<td>H</td>
<td>2-Me-butyl-S</td>
<td>36 (27-49)$^{f,1}$</td>
<td>1219 (1092-1361)$^{d,2}$</td>
<td>34</td>
<td>70% @ 1mg/mL</td>
</tr>
<tr>
<td>PRC 408</td>
<td>H</td>
<td>2-Et-butyl-S</td>
<td>37 (32-43)$^{d,1}$</td>
<td>5036 (4514-5618)$^{b,2}$</td>
<td>136</td>
<td>27% @ 1mg/mL</td>
</tr>
<tr>
<td>PRC 531</td>
<td>H</td>
<td>2-Et-hexyl-S</td>
<td>40760 (30940-53680)$^{b,1}$</td>
<td>3527 (2261-5503)$^{b,1}$</td>
<td>0.09</td>
<td>0% @ 1mg/mL</td>
</tr>
</tbody>
</table>

*Values in brackets are 95% CI. $S =$ mosquito selectivity as defined in Eqn 4.1. $\text{IC}_{50}$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 AgAChE 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 AgAChE 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 AgAChE 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 AgAChE 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 AgAChE inhibitory potency. Further elongation of the thioalkyl chain by a methyl group (PRC 407) yielded a 2-fold gain in AgAChE 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 AgAChE 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 AgAChE 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),
caused a > 1100-fold loss in AgAChE 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 C₂, 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.

![Chemical structures of and carbamoyl analogs of PRC 331 used in the study.]

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 AgAChE inhibitor potency or mosquito toxicity. In HuAChE however, we observed micromolar IC₅₀s 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 AgAChE 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

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>R₁</th>
<th>R₂</th>
<th>AgAChE-IC₅₀, nM</th>
<th>HuAChE-IC₅₀, nM</th>
<th>S</th>
<th>% mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC 507</td>
<td>H</td>
<td>Hex.</td>
<td>&gt;100,000</td>
<td>6293 (5126-7460)</td>
<td>&lt;0.06</td>
<td>4% @ 1mg/mL</td>
</tr>
<tr>
<td>PRC 565</td>
<td>Allyl carbamoyl</td>
<td>H</td>
<td>&gt;100,000</td>
<td>2835 (1719-4677)</td>
<td>&lt;0.03</td>
<td>0% @ 1mg/mL</td>
</tr>
<tr>
<td>PRC 566</td>
<td>Me-carbamoyl</td>
<td>H</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>N/A</td>
<td>4% @ 1mg/mL</td>
</tr>
<tr>
<td>PRC 567</td>
<td>i-But-carbamoyl</td>
<td>H</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>N/A</td>
<td>0% @ 1mg/mL</td>
</tr>
<tr>
<td>PRC 568</td>
<td>Di-Et-carbamoyl</td>
<td>H</td>
<td>&gt;100,000</td>
<td>2299 (1189-4448)</td>
<td>&lt;0.023</td>
<td>0% @ 1mg/mL</td>
</tr>
<tr>
<td>PRC 569</td>
<td>N-Et, N- Me-carbamoyl</td>
<td>H</td>
<td>&gt;100,000</td>
<td>5479 (2830-8128)</td>
<td>&lt;0.06</td>
<td>0% @ 1mg/mL</td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>AgAChE IC&lt;sub&gt;50&lt;/sub&gt;* nM</th>
<th>HuAChE IC&lt;sub&gt;50&lt;/sub&gt;* nM</th>
<th>S</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; or % mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC 331</td>
<td>tert-butyl</td>
<td>H</td>
<td>36 (34-38)&lt;sup&gt;a,g&lt;/sup&gt;</td>
<td>320 (293-347)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9</td>
<td>31 (14-60)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 505</td>
<td>-CF&lt;sub&gt;3&lt;/sub&gt;</td>
<td>H</td>
<td>&gt;10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/A</td>
<td>393 (333-471)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 515</td>
<td>tert-butyl-CF&lt;sub&gt;3&lt;/sub&gt;</td>
<td>H</td>
<td>345 (287-415)&lt;sup&gt;b,1&lt;/sup&gt;</td>
<td>2426 (1506-3346)&lt;sup&gt;b,1&lt;/sup&gt;</td>
<td>7</td>
<td>61 (43-80)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 394</td>
<td>tert-But.</td>
<td>para-Br</td>
<td>14 (12-17)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>48 (40-56)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.4</td>
<td>257 (239-276)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 553</td>
<td>tert-But.</td>
<td>ortho-Br</td>
<td>576 (260-892)&lt;sup&gt;b,1&lt;/sup&gt;</td>
<td>1223 (913-1533)&lt;sup&gt;b,2&lt;/sup&gt;</td>
<td>2</td>
<td>599 (570-627)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 506</td>
<td>tert-But.</td>
<td>para-I</td>
<td>2146 (1195-2309)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>638 (538-752)&lt;sup&gt;d,2&lt;/sup&gt;</td>
<td>0.3</td>
<td>20% @ 1 mg/mL</td>
</tr>
<tr>
<td>PRC 510</td>
<td>tert-But.</td>
<td>–CF&lt;sub&gt;3&lt;/sub&gt;ketone</td>
<td>7775 (6235-9317)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37 (22-52)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0048</td>
<td>8% @ 1 mg/mL</td>
</tr>
<tr>
<td>PRC 387</td>
<td>Me&lt;sub&gt;3&lt;/sub&gt;-Si-</td>
<td>H</td>
<td>50 (40-64)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>488 (452-526)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9.8</td>
<td>169 (162-176)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 524</td>
<td>Me&lt;sub&gt;3&lt;/sub&gt;-Si-</td>
<td>ortho-Me</td>
<td>378 (318-450)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2418 (2032-2814)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.4</td>
<td>241 (154-330)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 559</td>
<td>Me&lt;sub&gt;3&lt;/sub&gt;-Si-</td>
<td>meta-F</td>
<td>2622 (1260-5453)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4161 (3714-4616)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.6</td>
<td>4% @ 1 mg/mL</td>
</tr>
<tr>
<td>PRC 511</td>
<td>Me&lt;sub&gt;3&lt;/sub&gt;-Si-</td>
<td>-CF&lt;sub&gt;3&lt;/sub&gt;ketone</td>
<td>30950 (29220-32680)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>852 (732-972)&lt;sup&gt;d,2&lt;/sup&gt;</td>
<td>0.028</td>
<td>8% @ 1 mg/mL</td>
</tr>
<tr>
<td>PRC 540</td>
<td>tert-pentyl</td>
<td>H</td>
<td>35 (33-37)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>48 (37-60)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.4</td>
<td>68 (64-72)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 522</td>
<td>F&lt;sub&gt;3&lt;/sub&gt;C-butyl-2-yl</td>
<td>H</td>
<td>42 (38-46)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>104 (86-124)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5</td>
<td>115 (95-147)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 388</td>
<td>Et-Me&lt;sub&gt;2&lt;/sub&gt;-Si</td>
<td>H</td>
<td>72 (67-78)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>626 (515-761)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>8.7</td>
<td>185 (154-229)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 525</td>
<td>Et-Me&lt;sub&gt;2&lt;/sub&gt;-Si</td>
<td>ortho-Me</td>
<td>630 (556-714)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>1379 (1205-1559)&lt;sup&gt;b,2&lt;/sup&gt;</td>
<td>2.2</td>
<td>16% @ 1 mg/mL</td>
</tr>
<tr>
<td>PRC 547</td>
<td>Et-Me&lt;sub&gt;2&lt;/sub&gt;-Si</td>
<td>meta-F</td>
<td>189 (158-225)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>1941 (1649-2245)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10.3</td>
<td>439 (405-474)&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRC 512</td>
<td>Et-Me&lt;sub&gt;2&lt;/sub&gt;-Si</td>
<td>-CF&lt;sub&gt;3&lt;/sub&gt;ketone</td>
<td>25200 (24550-25862)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>844 (691-998)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.033</td>
<td>0% @ 1 mg/mL</td>
</tr>
</tbody>
</table>

*Values in brackets are 95% CI, *LC<sub>50</sub> values in µg/mL. ‘S’ denotes mosquito selectivity (Eqn 4.1)
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 AgAChE potency, compared to a 4-fold loss in HuAChE, relative to PRC 331. Consequent to this loss of AgAChE 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 AgAChE 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 AgAChE and HuAChE, respectively, when compared to PRC 331. Despite this potency loss, the compound is only half as toxic to mosquitoes. Flourination at the *meta* position of the trimethylsilyl group (PRC 559) caused a 50- and a 9-fold loss of potency to AgAChE and HuAChE, respectively and consequently rendered the compound non-toxic to mosquitoes (Table 4.4). Flourination 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 AgAChE 9-fold, compared to a 2-fold loss in HuAChE. This decreased AgAChE 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 AgAChE 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](image-url)  
**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

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

*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](image)

**Figure 4. 7.** Comparison of AgAChE 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.
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 AgAChE 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 HuAChE, 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 
AgAChE 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 AgAChE 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 AgAChE, 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 AgAChE. 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 \textit{in vivo} detoxication of \(N\)-methylcarbamates in insects occurred through P450-mediated hydroxylation of \(N\)-methylcarbamates to \(N\)-hydroxymethylcarbamates. Their investigations involved testing synergized and non-synergized \(N\)-substituted analogs of \(N\)-methylcarbamates such as \(N\)-hydroxyethylcarbamate, \(N\)-hydroxypropylcarbamate, \(N\)-hydroxybutylcarbamate and \(N\)-hydroxyhexylcarbamate 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 \(N\)-hydroxymethylcarbamates is not the major detoxication pathway and that unmethylated (\(N\)-methyl) 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, \textit{in vivo}. For instance, PRC 515, which has a trifluorinated methyl placed on the \textit{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 \(Hu\)AChE 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 \textit{whole} \textit{tert}-butyl side chain, as observed in drug metabolism (Hughes et al. 2010; Müller et al. 2007; Park et al. 2001). Change of \textit{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 AgAChE 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 AgAChE. 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 AgAChE 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 AgAChE.

4.4.6 Trifluoromethylketone replacement of carbamate moiety

A non-carbamate trifluoromethylketone analog of PRC 331 (PRC 510) had low AgAChE 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 AgAChE 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 trimethylsilyl analog was nearly half as potent to fly AChE, compared to the *tert*-butyl analog. In the current study, the trimethylsilyl 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\(^{\delta^+}\)C\(^{\delta^-}\) (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. Disubstitution by fluorination (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, tert-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 AgAChE using different patterns of chemical substitution.
References


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 (AgAChE-LiN) and mutant recombinant AgAChE whose I70 is substituted with Y70, to mimic the human phenotype (AgAChE-I70Y). I also used mosquito homogenates of G3-strain (AgAChE-G3; susceptible) and Akron-strain (AgAChE-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 AgAChE 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 \textit{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).

\textbf{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 *AgAChE-LiN* (N= normal, representative of WT recombinant *AgAChE*) and *AgAChE-I70Y* were supplied by Dr. Jianyong Li, of the Department of Biochemistry, Virginia Tech. Recombinant double *AgAChE* mutant M438P and D441Y and a single *AgAChE-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 *AgAChE-G119S* (Akron AChE), *AgAChE-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 AgAChE 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 AgAChE-I70Y and for any future comparisons with the recombinant AgAChE-LiN. Total protein quantified for the stock of recombinant AgAChE-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 AgAChE-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 °C and 405 nm.

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

^aExtinction coefficient of DTNB at 405 nm and 25 °C = 12800 M⁻¹ cm⁻¹
^bPath length = 0.5 cm, with 200 µL per well.
The Michaelis-Menten constant ($K_m$, Eqn. 5.2) and maximal velocities ($V_{max}$) 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®.

Michaelis-Menten equation: $V_o = \frac{V_{max} \times [S]}{(K_m + [S])}$

$V_o$ and $V_{max}$ are the initial and final enzyme velocities, respectively, and $[S]$ is the substrate concentration. $K_m$ is the substrate concentration at half of $V_{max}$, and is a Michaelis-Menten constant for every substrate and enzyme.

Quantification of recombinant $AgAChE$ 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 $AgAChE$-I70Y and for any future comparisons with the recombinant $AgAChE$-LiN.
5.3 Results

5.3.1 Inhibition of AChE by dimeric tacrines

Inhibition potency of the following AChE 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.

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

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).
Inhibition potency of \textit{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 AgAChE-G119S, AgAChE-I70Y and ChkAChE was highest with \textit{bis}(8)-, \textit{bis}(9)- and \textit{bis}(6)-tacrines, respectively. Overall, the difference between the least and most potent inhibitor was highest in AgAChE-I70Y (211-fold), followed by AgAChE-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 AgAChE-I70Y, 15-fold in AgAChE-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 AgAChE-I70Y, whereas AgAChE WT had the highest IC$_{50}$s. Note also that the mutation of I70Y in AgAChE changes the pattern of responses with \textit{bis}(n)-tacrines to more closely match that of HuAChE (Fig. 5.3). Longer tethers (i.e.) \textit{bis}(10-12) show a loss in potency in all species except chicken, whose potency remains similar at \textit{bis}(9-12), creating a ‘plateau’ region. For each case, either THA or \textit{bis}(2)-tacrine was the least potent inhibitor. Maximal potency of all AChEs is observed between \textit{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 AgAChE-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 (AgAChE-G119S), G3 mosquito (AgAChE-WT), and chicken brain (ChkAChE) homogenates, and three recombinant AChE types namely; mosquito WT enzyme (AgAChE-LiN), AgAChE-I70Y, and HuAChE. The enzymes tested have different structural motifs at both the peripheral (e.g., AgAChE-I70Y) and catalytic sites (e.g., AgAChE-G119S).
Figure 5.3. Inhibition potency of peripheral site ligands to AChEs. Letters denote: HuAChE (A), AgAChE-I70Y (B), AgAChE-LiN (C), ChkAChE (D), WT AgAChE-G3 (E) and Akron AgAChE-G119S (F).
In Fig. 5.3, similar inhibition patterns are observed between HuAChE and AgAChE-I70Y, and also between AgAChE-LiN and WT AgAChE-G3. Although AgAChE-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\textsubscript{50}s generated by sigmoidal plots in Fig 5.3 were presented in Table 5.2 for statistical comparison. Potency of PRC 472 to AgAChE-LiN was 44-fold higher than in AgAChE-I70Y, whereas the difference between AgAChE-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 AgAChE-G119S is ca. 85-fold lower than that of BW284c51, while the difference in AgAChE-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 AgAChE-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.

<table>
<thead>
<tr>
<th>AChE Source</th>
<th>Inhibitor IC&lt;sub&gt;50&lt;/sub&gt;, nM (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRC 472</td>
</tr>
<tr>
<td>HuAChE</td>
<td>12,470 (11800-13180)&lt;sup&gt;a,1&lt;/sup&gt;</td>
</tr>
<tr>
<td>AgAChE-I70Y</td>
<td>3,046 (2668-3479)&lt;sup&gt;b,1&lt;/sup&gt;</td>
</tr>
<tr>
<td>AgAChE-LiN</td>
<td>70 (63-78)&lt;sup&gt;c,1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ChkAChE</td>
<td>26,540 (24180-29130)&lt;sup&gt;d,1&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT AgAChE-G3</td>
<td>166 (152-182)&lt;sup&gt;e,1&lt;/sup&gt;</td>
</tr>
<tr>
<td>AgAChE-G119S</td>
<td>4,904 (4565-5268)&lt;sup&gt;f,1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*RAR denotes Residual Activity Remaining, at 100,000 nM; the highest inhibitor concentration tested. IC<sub>50</sub> 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 $K_m$ and $V_{max}$ values were determined for four enzyme phenotypes; HuAChE, AgAChE-I70Y, AgAChE-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 AgAChE-LiN and AgAChE-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 $V_{max}$ with Chicken, followed by mosquito and lastly human AChE (Table 5.5). Lowest $K_m$ value for ATChI was obtained with AgAChE-LiN followed by ChkAChE, HuAChE and AgAChE-I70Y, in that order.

![Graph showing substrate velocities of AChEs with ATChI.](image)

**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.
Table 5.3. Kinetic properties of human, mosquito and Chicken AChEs

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Km; µM</th>
<th>Vmax; U</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuAChE</td>
<td>311 (237-385)_{a,c}</td>
<td>0.158 (0.148-0.168)_{a}</td>
</tr>
<tr>
<td>AgAChE-I70Y</td>
<td>358 (327-389)_{a}</td>
<td>0.238 (0.232-0.244)_{b}</td>
</tr>
<tr>
<td>AgAChE-LiN</td>
<td>199 (185-213)_{b,d}</td>
<td>0.228 (0.224-0.232)_{b}</td>
</tr>
<tr>
<td>ChkAChE</td>
<td>211 (159-262)_{c,d}</td>
<td>0.289 (0.272-0.306)_{c}</td>
</tr>
</tbody>
</table>

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 TcAChE sequence.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Excerpt from Multiple Sequence Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo</td>
<td>G---VVDA TTFQSVCYQVDTLYPGFEGHLMNPANEDCSSCLYLVWTVTPRTSTPFP 114</td>
</tr>
<tr>
<td>Bos</td>
<td>G---VLNATAFQVYQZTVDTLYPGFEGHLMNPANEDCSSCLYLVWTVTPRTSTPFP 111</td>
</tr>
<tr>
<td>Rattus</td>
<td>G---ILDA TTFTQVSVYQZTVDTLYPGFEGHLMNPANEDCSSCLYLVWTVTPRTSTPFP 114</td>
</tr>
<tr>
<td>Gallus</td>
<td>G---IRDAQTFTQTQVTVDTLYPGFEGHLMNPANEDCSSCLYLVWTVTPRTSTPFP 116</td>
</tr>
<tr>
<td>Torpedo</td>
<td>G---VVNA TTPNQCVTVDEQPGFSGHEMNPANEDCSSCLYNIVWSPPRTST- TV 111</td>
</tr>
<tr>
<td>Anopheles</td>
<td>G---VLNT TTPNCSQIVDTVFDFPQATMNFPNLSSCLE LVNVWAPRPFPKA-AV 111</td>
</tr>
<tr>
<td>Blattela</td>
<td>HHEGYNATMPNSCQIQDTVFDFPQALIVNFPTLSSCLELVNVWAPRPFPKA-AV 114</td>
</tr>
</tbody>
</table>

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

The AgAChE 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 $\pi$-$\pi$ 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-$\pi$ 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 $\pi$-$\pi$ 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 AgAChE.

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 AgAChE 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 AgAChE-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 AgAChE-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 \( \text{bis}(5) \)-tacrine and \( \text{bis}(7) \)-tacrine (Anderson et al. 2008; Muñoz-Ruiz et al. 2005; Wang et al. 1999) Comparison of HuAChE and the AgAChE-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 AgAChE-Akron (G119S), but not with G3 AgAChE WT. A striking feature of the Akron AChE is the subtle gain in potency with THA up to \( \text{bis}(5) \)-tacrine, as opposed to equivalent greater potency enhancement with all other AChEs. Quantitatively, net potency gain between THA and \( \text{bis}(5) \)-tacrine for AgAChE-G119S was ca. 4-fold, compared to 42-fold and 21-fold gain for AgAChE-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 AgAChE-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 > AgAChE-I70Y > AgAChE-G119S > ChkAChE > AgAChE WT. This trend shows another close association of HuAChE with AgAChE-I70Y. There is no clear explanation of the differences in net potency gain observed between AgAChE-G119S and WT AgAChE, 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, AgAChE-G3 and AgAChE-G119S by propidium and ethidium. Interestingly, BW284c51 is equipotent to the 3 enzyme phenotypes; HuAChE; AgAChE-I70Y and AgAChE-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 AgAChE-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 AgAChE-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 AgAChE-G119S compared to WT AgAChE-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 AgAChE-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 corroborates 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 \( TcAChE \). 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 \textit{Drosophila}.

5.4.3 I70Y decreases substrate affinity in AgAChE but has no significant effect on catalytic efficiency

Comparisons of AgAChE-LiN and AgAChE-I70Y substrate kinetics with ATChI revealed that substrate affinity decreases significantly in I70Y, but their maximal velocities (Vmax) are not significantly different. Interestingly, the \( K_m \) values of both HuAChE and AgAChE-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 AgAChE-LiN had the lowest \( K_m \) value (highest affinity for ATChI), the increased substrate affinity again did not correspond to faster maximal reaction rates. \( K_m \) value obtained for AgAChE-LiN is 2-fold higher than that of a purified recombinant AChE of the greenbug, \textit{Schizaphis graminum} (Zhao et al. 2010), indicating low variability in affinity for ATChI with these insect AChEs. Previous
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 $K_m$ values for a recombinant AgAChE with ATChI was approximately 64 $\mu$M. Their value was 3-fold lower than what I obtained in the current study, with AgAChE-LiN. Observed disparities in $K_m$ 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 AgAChE-LiN and AgAChE-I70Y were unpurified cell lysates. Since the primary focus of my objective was not to exhaustively characterize AgAChE-LiN, a more conclusive comparison of enzyme substrate kinetics between Jiang’s recombinant enzyme and AgAChE-LiN, which both share the same phenotype, will be reported elsewhere. Detailed comparative enzyme kinetics of HuAChE, AgAChE-LiN and AgAChE-I70Y with several substrates as well as inhibition by a wider array of catalytic and peripheral site ligands will be addressed in the future.
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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 \textit{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 \textit{Drosophila} and cockroach AChE, sensitivity to \textit{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 \textit{Drosophila} AChE display a high tether length dependent potency enhancement, unlike mosquito and cockroach AChE, a trend that partitions similarly with
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
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
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 \textit{in vivo}, the observed toxicity of PRC 515, a compound having low anticholinesterase activity, \textit{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 C₃. Future exploration of mosquito-selectivity with thioalkyl-substituted compounds should not exceed C₃.

An ethyl-S-phenyl substituted N-methylcarbamate (PRC 529) was potent to AgACHE, 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 \textit{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 \textit{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 AgAChE 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 AgAChE-I70Y, AgAChE0LiN, AgAChE-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

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Appendix B

Date: Thu, 03 Mar 2011 01:13:01 +0200 [03/02/2011 05:13:01 PM EDT]
From: Prof. Joel L. Sussman <joel.sussman@weizmann.ac.il>
To: James Mutunga <jmutunga@vt.edu>
Cc: Prof. Joel L. Sussman <joel.sussman@weizmann.ac.il>, Dr. Dawn M. Wong <dawnwong@vt.edu>
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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

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

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I am a PhD student at Virginia Tech, working with Dr. Dawn Wong who is a co-author of the paper. I would use
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