CHARACTERIZATION OF INSECT ACETYLCHOLINESTERASE ENZYME: DMSO-MEDIATED ALLOSTERIC EFFECTS, INHIBITOR PHARMACOLOGICAL PROFILE, AND ROLE IN THE NEUROTOXICITY OF INSECT REPELLENTS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2012
To my family (Mom, Dad, Adam, Emily, and Katelyn), for their encouragement and steadfast support throughout my journey at the University of Florida. I would also like to thank my advisor Dr. Jeff Bloomquist for all of his support and friendship he has provided me during my time at UF. To my committee members, Drs. Uli Bernier, Chelsea Smartt, Paul Lindser, and Maureen Long, thank you for your helpful guidance throughout my academic career.

To my wonderful and beautiful fiancée, thank you for helping me through the many trials and tribulations throughout my Ph.D. You made this journey fun and kept it exciting. To the AMAZING Bloomquist lab (Lacey Jenson, James Mutunga, Fan Tong, Nick Larson, Boonan Su, Rafique Islam, and honorary member Chris Holderman), thank you for your help within the lab, but more importantly, your friendship. Y’all have been a vital part of my positive experiences in Gainesville and I’m looking forward to many more elsewhere!
ACKNOWLEDGMENTS

My dissertation project was funded by a grant from the National Institute of Allergy and Infectious Disease, (R01 AI082581). Collaborations have been an extensive part of my project and include the USDA-CMAVE (Gainesville, FL), USDA-ARS (Kerrville, TX), Molsoft (LaJolla, CA), and Virginia Tech Chemistry Department (Laboratory of Paul Carlier).
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December 2012

Chair: Jeffrey R. Bloomquist
Major: Entomology and Nematology

Vector borne diseases are of great importance, and there has been significant interest in the design of novel mechanisms for the control of insect vectored diseases. Utilization of primarily one chemical class of insecticides for disease vector control has increased insecticide resistance, limiting the effectiveness of currently utilized control methods within malaria endemic regions. I first report on the enzyme kinetic effects of a unique allosteric solvent interaction that occurs between mosquito-selective carbamates, dimethyl sulfoxide, and Anopheles gambiae acetylcholinesterase. These results have implications for the high throughput screening of insecticides, as continued use of current methods using DMSO likely facilitates the overlooking of lead compounds. Secondly, I present a characterization of the inhibitor profile of acetylcholinesterase from Boophilus microplus (Bm) and Plebotomus papatasi (Pp) compared to human and bovine acetylcholinesterase, in order to identify divergent pharmacology that could lead to selective inhibitors. Results indicate a unique structure of Bm acetylcholinesterase that could lead to the design of novel inhibitors to replace the currently utilized class of insecticides. Additionally, Bm and Pp display low nanomolar sensitivity to a variety of newly designed carbamate insecticides that could provide an excellent lead compound
for vector control. Thirdly, I report an analysis of the mode of action of the toxicity to the insect repellent \(N,N\)-Diethyl-meta-toluamide (DEET). Recent reports suggest DEET is an acetylcholinesterase inhibitor, potentially causing a toxicity risk in exposed human individuals. Results indicate that DEET is a poor acetylcholinesterase inhibitor, but instead mimicks the action of octopamine on the insect central nervous system. Further analysis found a blocking action on mammalian neuronal sodium and potassium channels similar to local anesthetics, such as lidocaine. Such an action would explain the numbness caused by DEET when applied to human skin. Overall, I conclude that toxicity of DEET through anticholinesterase properties is unlikely and would be of little relevance to human safety. Lastly, the evaluation of mosquito-selective carbamates to mosquito vectors and agricultural pests were reported. Results indicate that our novel carbamates possess unusual insect selectivity that has implications for mitigation of mosquito insecticide resistance due to agricultural uses, particularly in malaria endemic regions.
CHAPTER 1
LITERATURE REVIEW

1.1 Mosquito Borne Vectors

Vector borne diseases are either emerging or resurging due to a variety of different factors including, but not limited to, consequences of public health policy, insecticide resistance, and the change from eradication of vectors to emergency response (Gubler, 1998a). This change from prevention to emergency response allows diseases to thrive and continuously infect humans versus eliminating infections through eradication. In the early 1900’s, it was realized that the mosquito was capable of transmitting disease from human to human, making the design of control programs to manage the arthropod vector of critical importance (Gubler, 1998a). By the 1960’s, urban yellow fever and dengue fever were controlled in Central and South America and were eliminated in North America through destruction of breeding sites and controlled insecticide usage (Gubler, 1998a). Similarly, malaria was greatly reduced in the Americas, Asia, and the Pacific Islands through control programs combining insecticide spraying and elimination of breeding sites (Gubler, 1998a).

With the exception of Antarctica, mosquitoes are capable of thriving in many biotic communities such as tropical forests, salt marshes, and the tundra (Mullen and Durden, 2002). Due to their ubiquitous presence, high potential for domestication, inhabitance of many ecosystems, and ability to acclimate to a change in environment and in some cases to shift host preference, mosquitoes are the most important arthropod affecting human health. Among these is the Anopheles gambiae (Giles; An. gambiae) mosquito. This species is regarded as the world’s most important parasitic infection in humans (White, 1996) as it results in 1.5 -3 million deaths annually (WHO, 2003). Despite rigorous control efforts, over 100 countries are at risk for malaria, making it the major cause of mortality in third world countries (WHO, 2003).
Anopheles spp. mosquitoes are found in many countries throughout the world, allowing for the widespread presence of the malaria parasite. An. gambiae is considered to be the primary malaria vector in Sub-Saharan Africa (Coluzzi, 1984), An. darlingi is the primary vector in Latin America (Conn et al., 2006), and An. dirus is considered to be the primary vector in Southeast Asia (Manh et al., 2010). Although the aforementioned species are considered to be primary malaria vectors, secondary vectors have been documented to cause substantial malaria transmission. Secondary vectors include: An. arabiensis, An.funestus, An. nili, and An.moucheti. Ae. aegypti (Linn.) and Ae. albopictus (Skuse) are also major vectors of several important diseases in humans. Dengue fever and dengue-dengue hemorrhagic fever (DHF) are considered to be the most important tropical infectious diseases, after malaria (Gubler, 1998b). Ae. aegypti is considered to be the most efficient dengue vector due to its preference for human hosts and resides in densely populated locations. However, Ae. albopictus is considered to be the primary vector in some locations of the world in which Ae. aegypti populations are low. Ae. aegypti is mainly found in the sub-tropical zone of the Americas, and is a great threat to humans due to domestication and their diurnal habits. Additionally, Ae. species have been found to transmit various other diseases such as West Nile, LaCrosse encephalitis, and Yellow Fever (Watts et al., 1973; WHO, 1997; Nash et al., 2001) viruses.

Other mosquito genera, such as Culex (Cx.), are also known to act as vectors for numerous other diseases that affect thousands of people worldwide. Cx. quinquefasciatus (Say) is debatably the primary African vector within the genus, and has been observed in staggering densities. In Rangoon, Myanmar, this particular species has been estimated to have densities of 15 million per square kilometer resulting in 80,000 bites per year/per person (Mullen and Durden, 2002). Cx. quinquefasciatus are inhabitants of many locations throughout the world
including sub-Saharan Africa, a nearly identical range to An. gambiae. Over a billion people in
as many as 80 countries are at risk for lymphatic filariasis (LF), which is vectored by Cx.
quinquefasciatus (WHO, 2000). Cx. quinquefasciatus is a nocturnal mosquito and feeds
opportunistically on mammals, which can be an important consideration for control mechanisms
(Mullen and Durden, 2002). Due to the high number of infections from multiple vectors and
omnipresence of mosquitoes, control programs are vital for reduction of disease.

1.2 Mosquito Borne Diseases

Malaria is considered to be the most important insect transmitted disease; it affects 300-
500 million individuals with approximately 1.5-3 million fatalities annually (Giles and Warell,
1993; WHO, 2003). The malaria parasite, Plasmodium falciparum, is vectored by An. gambiae
(Giles), and has increased morbidity/mortality towards individuals with compromised immune
systems, including children and pregnant women (Greenwood et al., 1987). In the United
States, endemic malaria has been eradicated, even though the Anopheles spp. vectors are still
present. This eradication was achieved through the use of synthetic pesticides, and through
improved socio-economic conditions such as window screens and air conditioners (Williams,
1963; Zucker, 1996). Malaria control programs have been implemented worldwide and have
been successful in eradicating malaria within developed countries. However, developing or
underdeveloped countries have not been able to eradicate the disease for multiple reasons
including, but not limited to financial burdens, poor compliance of control programs, and
insecticide resistance. Control programs are varied throughout the world and have a large
influence on the impact of malaria within a particular region.

Dengue fever and DHF have increased substantially over the past 40 years and in 1996
over 40% of the worlds’ population resided in an area deemed at risk by the World Health
Organization (WHO, 1997). Within the past 10 years there have been an estimated 100 million
cases of dengue fever, 500,000 cases of dengue hemorrhagic fever, and approximately 25,000 deaths annually (Gubler, 1998b). Although dengue fever has been an important tropical disease worldwide for many years, it is now becoming an eminent threat within the United States. Dengue is often considered a disease found within tropical and subtropical locations, but it is capable of being transmitted in temperate climates as well. For approximately four decades (1940 – 1980), there were no reported cases of acquired dengue fever within the continental United States. However, environmental factors, competent mosquito vectors, increased outdoor activities, and reduced control measures within the United States provided conditions adequate to facilitate a dengue outbreak within the United States. Confirmation of this possibility is shown by locally acquired cases of dengue in 1980 along the Texas-Mexico border. These cases are thought to have coincided with large outbreaks in neighboring Mexican cities. Furthermore, since 2009, there have been 61 confirmed cases of locally acquired dengue fever in Key West Florida alone (CDC, 2010), and according to the Miami-Dade Health Department, two locally acquired dengue cases in Miami-Dade county. This northward spread of the disease toward larger cities in Florida has increased the concern that dengue could reemerge as an endemic disease in the U.S. (Vaidyanathan, 2010).

There are a number of factors that have been identified as reasons for the reintroduction of dengue in the U.S. First, dengue was added to the list of Nationally Notifiable Infectious Conditions within the past year (2010) (Franco et al., 2010). Due to this, awareness within the medical community is low and can potentially lead to delayed identification of dengue outbreaks. Secondly, increased world travel allow for dengue outbreaks to more likely due to movement of the virus and the vectors. Thirdly, the overall range of the two vectors, *Ae. aegypti* and *Ae. albopictus*, are continuing to expand and will potentially lead to dengue outbreaks within the
United States. Currently, *Ae. albopictus* has been found to inhabit locations from the eastern seaboard to New England and the Mississippi River up to Chicago. Lastly, governmental agencies have had severe budget cuts, which prevent adequate vector surveillance and control programs to be established, thus minimizing the country’s ability to respond and control future outbreaks (Franco et al., 2010).

Currently, the Florida Keys utilize a variety of the aforementioned control techniques to minimize the transmission rates of dengue fever. The Mosquito Control Association within the Florida Keys actively practices source reduction via door – to – door campaigns to isolate and eliminate mosquito breeding sites. Larval control is accomplished through the application of larvicides such as spinosad or *Bacillus thuringiensis israelensis*. When applied properly, these larvicides have no effect on non-target organisms and are considered to be the optimal method for controlling mosquito populations in the Florida Keys. However, when populations are too large for larval control, the spraying of adulticides on adult populations has become the principal method for controlling the disease vectors. According to the Florida Keys Mosquito Control Association, vehicles or airplanes spray a fine mist of insecticides to locations with adult mosquito populations through an Ultra-Low Volume (ULV) method. The ULV method deploys less than an ounce of insecticide per acre. When performing ground adulticide treatments, it is common for the county to utilize permethrin as the control agent whereas organophosphates are used in the aerial treatments. Although these insecticides are administered in extremely low amounts, public outcries often limit the use of these effective control measures.

Control of mosquitoes through the use of insecticides is complicated due to the large abundance and diversity of environmentally sensitive ecosystems found within the Florida Keys. There are a large number of marine sanctuaries, refuges, and national parks that convolute the
problem of controlling mosquito vectors. One organism of concern is *Cyclargus thomasi bethunebakeri*, or the Miami Blue Butterfly (MBB). The MBB is a coastal butterfly that inhabits sunny areas at the edge of tropical hardwood forests (Zhong, 2007). This insect has a total developmental time of approximately 30 days, which yields them more susceptible to a number of insecticidal sprays in the field. This species of insect is considered to be one of the rarest in North America and was declared to be critically endangered by the state of Florida in 2002. Currently, Bahia Honda Key State Park has the only isolated population of MBB that still flourishing in the wild (Center of Biodiversity, 2005) and there is significant controversy over utilizing insecticides for mosquito control near potential MBB habitats. Studies have found residues of naled and fenthion (organophosphates) within the hammock ecosystems of wildlife refuges in the state of Florida due to unintentional insecticide drift from aerial applications. Recently, research has been performed to determine the effects of organophosphates toward MBB larvae when using ULV for mosquito control. Data suggests that the overall survival rate of MBB larvae is 73.9% when exposed to ULV (1000 μg/m²) organophosphates in the spray zone. This survival rate is significantly lower than for MBB exposed in them drift zone (90.6%) or control zones (100%), indicating that mortality to the MBB larvae occurs within the mosquito targeted spray area. However, these data also suggest that mortality of MBB is minimal outside of the targeted spray area (Zhong, 2007).

The results of the previous study indicates the dire need for the development of a novel insecticide that can be used for dengue control in the state of Florida, and similar locations worldwide, with minimal to no mortality to non-target species, including the Miami Blue Butterfly. Development of such an insecticide could be essential for a successful mosquito control program while allowing populations of the endangered species to survive.
1.3 Other Invertebrate Disease Vectors and Their Respective Vectored Diseases

There are a number of other medically important disease vectoring arthropods that impact human and animal health through the transmission of disease and the economic burden the diseases produce. One example is Phlebotomine sandflies, which are nematoceran insects that have the capability of transmitting a number of human and animal related diseases. Sandflies are most commonly known to transmit leishmaniasis, a potentially disfiguring disease that affects people in more than 80 countries worldwide (Desjeux, 1996). Leishmaniasis is considered to be an emerging and uncontrolled disease as it is endemic in nearly 100 countries with nearly 350 million people at risk (WHO, 2007, 2010). *Phlebotomus papatasi* is the primary species of the old world that is known to vector *Leishmania major*, the causative pathogen for zoonotic cutaneous leishmaniasis (Jaffe et al., 2004; Kravchenko et al., 2004). *Phlebotomus papatasi* populations are widespread with endemicity in central Asia, India, North and Central Africa, and the Middle East (WHO, 2007). This high geographic prevalence combined with the biology of the sandfly make this insect exceptionally difficult to control. Burrowing rodents are reservoir hosts for *L. major* and sand flies utilize rodent burrows for various insect lifestages, making the control of these insects through direct insecticidal application difficult (Schlein and Muller, 2010; Wasserberg et al., 2011; Mascari et al., 2012).

Ticks are also well-known arthropods of medical and veterinary importance as they are disease vectors to both, humans and animals. Approximately ten percent of the 867 tick species are known vectors of numerous pathogens that infect humans and animals. These tick vectored diseases result in significant economic losses and mortality directly due to their feeding behavior (Jongejan and Uilenberg, 2004) of livestock. Ticks transmit a greater diversity of pathogenic organisms, protozoa, and viruses than any other arthropod (Jongejan and Uilenberg, 2004). The global importance of ticks is primarily through the diseases transmitted to livestock, as they are a
The major constraint of livestock production and therefore, economic growth is the transmission of diseases. The major tick-borne diseases that affect livestock include babesiosis, anaplasmosis, theileriosis, and heartwater. Beyond the transmission of diseases, ticks are capable of negatively affecting livestock and their economic value through extensive blood loss, reduced value of skins/hides, and reduced milk production (Jongejan and Uilenberg, 2004). An agriculturally important tick that affects the U.S. population is the cattle tick, *Rhipicephalus (Boophilus) microplus* (Canestrini; *Bm*). This species of tick is a potentially deadly pest of cattle as they are primary vectors for *Babesia*, a protozoan parasite that causes the deadly hemolytic disease known as babesiosis. Due to the high value of livestock and the increasing difficulty to control tick vectors, there is a need for the development of insecticides with novel modes of action that can augment the currently utilized control methods to continue reducing disease vector populations.

### 1.4 Vector Borne Disease Control Methods

Control methods vary substantially in cost, sustainability, applicability, and effectiveness; however, the principle remains the same: reduction of morbidity and mortality of vector borne diseases through reduction of transmission levels, which in turn can potentially reduce the severity of infection (Bay, 1967). Prior to the use of dichloro-diphenyl-triphenylethane (DDT), the majority of vector control was targeted toward the larval stages, which requires extensive knowledge of insect behavior and ecology (Brogdon and McAllister, 1998). Larvicides are still commonly used, however a combination of control methods are now used. In 1940, the development of DDT revolutionized vector control by allowing effective control measures to be introduced into mosquito-laden areas through indoor residual spraying (IRS). The use of DDT in malaria endemic regions assisted in eliminating the disease in the United States and Europe, and reduced transmission by up to 99% in Sri Lanka, India (Attaran and Maharaj, 2000). Despite its positive results on vector control, DDT was banned due to environmental harm, high persistence.
in vegetation and mammals, and potential carcinogenic/teratogenic properties towards humans (Roberts, 1997; Turusov et al., 2002). This ban has resulted in researchers attempting to develop mosquitocides that are less persistent, more selective, have minimal side effects, and are cheaper to produce (Carlier et al., 2008; Berg, 2009).

The World Health Organization (WHO) has focused on malaria reduction in sub-Saharan Africa by controlling the disease vectors with the use of two primary methods. Indoor residual spraying (IRS) has been utilized by spraying a persistent insecticide in the house interior and eaves of houses. Although utilization of IRS has had great success in decreasing the concentration of malarial vectors, there has been a decline in the use of IRS due to lack of funding from local governments, concerns of environmental harm, and potential human intoxication (WHO, 2006). The second form of controlling malarial vectors is by administering long-lasting insecticide-treated bed nets (ITNs), usually treated with a pyrethroid (WHO, 2006). There have been several studies that report reduced malaria infection due to reduction of insect vectors through the use of ITNs (Choi et al., 1995; Curtis et al., 1998).

Lymphatic Filariasis is vectored by *Cx. quinquefasciatus* and more effective means of control are needed for this vector. Currently, minimization of cases for this disease is based around reduction of mosquito numbers, but also incorporates treatment of the microfilaria through medicinal compounds such as albendazole (Ottesen et al., 1999). Adult *Cx. quinquefasciatus* control programs are very similar to those of *Ae. aegypti* and *Ae. albopictus*, but there is a greater utilization of biopesticides like *Bacillus sphaericus* (Barbazan et al., 1997). A major problem with chemical control of *Cx. quinquefasciatus* is the increased prevalence of resistance. Continued widespread use of malathion, an organophosphate, has resulted in a broad spectrum of insecticide resistant *Cx. quinquefasciatus*, whereas *Ae. aegypti* with an identical
range and exposure rate showed no resistance (Magdalena et al., 2000). This greater resistance potential suggests the need for reduced broad application of insecticides to a more narrow use, such as control through ITNs. *An. gambiae* and *Cx. quinquefasciatus* possess common nocturnal feeding habits, making it feasible to jointly control malaria and filariasis through ITNs. This method is currently successful and has products such as permethrin-incorporated Olyset Net® bed nets. In a recent study of these nets, they resulted in increased exophily of *Cx. quinquefasciatus* that factored into feeding success rates of 14%, to 15% compared with 35% when untreated nets were used (Guessan et al., 2008). As with other mosquito vectored diseases, there is a need for new, selective mosquitocides for continued reduction of lymphatic filariasis.

Dengue fever, transmitted by *Ae. aegypti* and *Ae. albopictus* has no vaccine, and therefore the only way to reduce disease transmission is to control the primary vectors (Gubler, 1989). These mosquito species are domesticated and have evolved to breed in water-laden containers of relatively small volume, such as used car tires, old plastic cartons, and flower vases at cemeteries (Christophers, 1960). Plastic containers are the primary breeding sites for *Aedes* spp., which impacts control measure strategies (Vezzani and Schweigmann, 2002). Control of dengue fever through the reduction of *Ae. aegypti* and *Ae. albopictus* begins with the adequate covering of plastic containers (ie: cemetery vases, used car tires, etc.) to prevent access to egg-laying females (Vezzani and Schweigmann, 2002). Secondly, biological control, although not commonly utilized, has been used to control the two dengue fever vectors (Turley et al., 2009). In lieu of biological control, many countries have begun control with natural and chemical larvicides and have had success in reducing the number of vector mosquitoes (Garcez et al., 2009). There are a number of proven larvicides such as deltamethrin, temephos, DDT, methoprene, and *Bacillus thuringiensis* subsp. *Israelensis*, as well as other botanical larvicides.
(Kumar et al., 2009; Borovsky, 2003). However, prolonged use of several aforementioned synthetic larvicides has led to resistance and therefore decreased control (Mulla et al., 2004; Kroeger et al., 2006). Control of adult mosquitoes includes broad applications of insecticides via aircraft, vehicles, and by hand (WHO, 2008). These techniques result in satisfactory levels of adult mosquito control that often persist through the peak dengue transmission period (Gratz, 1991). Although these methods are effective for controlling mosquito vectors, the broad application of insecticides could have deleterious effects of non-target organisms and will likely increase the presence of insecticide resistance. Due to these factors, there is a need for the design of new, selective mosquitocides for the control of disease vectors worldwide.

Non-mosquito disease vectors are also of critical importance as these arthropods may also be competent in vectoring deadly human and animal diseases. Control of phlebotomine sandflies is very similar to the control methods used for An. gambiae. The use of ITNs are used primarily for malaria control, but their effectiveness on phlebotomine sandfly populations has been evaluated in numerous different countries and shown some promise for control (Maroli and Lane, 1989; Mutinga et al., 1992; Basimike and Mutinga, 1995). However, the effectiveness of ITNs may be limited because the highest sandfly biting activity is during the twilight hours, usually before people are outdoors. Due to this crepuscular activity, control has shifted to utilizing IRS methods and insecticide impregnated curtains hung across potential sites of entry (ie: windows and doors) (Alexander and Maroli, 2003). Studies have shown that curtains impregnated with permethrin significantly reduced the human biting rate and resting density of P. papatasi, indicating a potential control method for reduction of vectored disease from phlebotomine sandflies (Elnaiem et al., 1999). Recently, research has been performed to determine the effectiveness of targeting sandflies through rodents, their primary host for
bloodfeedings (Mascari et al., 2012). Current sandfly control methods could be substantially augmented through the development of a feed through insecticide that possesses high sandfly toxicity, yet low mammalian toxicity.

Control of tick-vectored diseases is different than control of dipteran vectors due to the different hosts and the different developmental habitats. Control programs for the cattle fever tick also utilize insecticides, but application of these chemicals is usually applied directly to the host animal through sprays and dips versus aerial sprays or static control methods. Although *Boophilus microplus* (*Bm*) has been eradicated within the United States, Mexican cattle still suffer infestations of *Bm* that harbor *Babesia* spp. and pose a threat to cattle populations within the United States through reintroduction (Graham and Hourrigan, 1977; Bram et al., 2002). Reintroduction of infected *Bm* from Mexico into the United States is of extreme concern due to the large number of imported cattle and also due to the likelihood of infected ungulate wildlife crossing the Rio Grande River and entering southern Texas (George, 1990).

To prevent the return of *Bm* to the United States, the United States Department of Agriculture (USDA) implemented the Cattle Fever Tick Eradication Program (CFTEP) which mandates a quarantine zone, dipping of all imported cattle into 0.3% - 0.25% coumaphos, and a 7-14 day quarantine period (Graham and Hourrigan, 1977; Miller et al., 2005). Although CFTEP has been effective in reducing *Boophilus* populations, control has become increasingly difficult due to escalating organophosphate (OP) and pyrethroid resistance of *Bm* within Mexican cattle (Miller et al., 2005; Li et al., 2003; Rosario-Cruz et al., 2005; Baxter and Barker, 1998).

### 1.5 Mode of Action of Insecticides Used for Vector Control

Insecticides continue to be a mainstay for mosquito control programs utilizing the integrated vector management approach for the control of vector borne diseases (Hemingway
Acetylcholinesterase (AChE, EC 3.1.1.7) inhibitors block the hydrolytic action of AChE, and carbamate (CB) and organophosphate (OP) compounds are two classes of insecticides commonly known to inhibit this enzyme (Bloomquist, 1999). Figure 1-1 depicts common CBs (Bendiocarb and Propoxur) and OPs (Malathion and Fonofos) used for vector control. The OPs are a group of insecticides that are chemically diverse and are classified based on the elements attached to the central phosphorous atom. Due to the majority of OPs being phosphorothionates, cytochrome P450 monooxygenases within the insect must bioactivate them through a reaction known as oxidative desulfuration (Bloomquist, 1999). Carbamates are a similar group of insecticides when compared to OP’s as they are both anticholinesterases and induce similar signs of intoxication. Carbamates are all esters of carbamic acid that often posses an aryl group as the leaving group. The insecticidal effect of CBs and OPs is similar due to their inherent ability to inhibit acetylcholinesterase (AChE). AChE is a serine hydrolase needed for regulating the synaptic action of the neurotransmitter acetylcholine (Ach). The AChE-directed insecticides react with a serine residue that is located at the catalytic site found within the AChE gorge (Fukuto, 1990). The carbamoylated or phosphorylated enzyme is no longer able to hydrolyze ACh, resulting in the buildup of ACh in the nerve synapse (Cohen and Oosterbaan, 1963). This effect causes excessive excitation of the nerves, producing uncoordinated movements, tremors, and paralysis (Yu, 2008). Although these two classes of insecticides are very similar in their mode of action, they do possess notable differences. The phosphorylation of AChE is considered to be irreversible and can inactivate the enzyme for hours to days whereas carbamoylation of
AChE is less stable and will hydrolyze with a half-life of approximately 40 min (Bloomquist, 1999).

A second commonly used class of insecticides for vector control is the pyrethroids. Pyrethroids are synthetic compounds developed from pyrethrins found in flowers of *Chrysanthemum cinerariaefolium*. The compounds within this class are typically esters of chrysanthemic acid (Bloomquist, 1999). The compounds within this insecticidal class target the voltage-gated sodium channels of insects (discussed in further detail in a separate section) and possess greater selective toxicity for insects when compared to the OP’s. The high efficacy and selectivity of pyrethroids has resulted in their wide utilization as pest control agents (Bloomquist, 1999). Pyrethroids are classified into two types, type 1 or 2, based on their alcohol substituent. More specifically, type 2 pyrethroids are commonly classified by the presence of an alpha-cyano group, whereas type 1 pyrethroids do not possess this functional group, and are therefore approximately 10-fold less toxic to insects (Bloomquist, 1999). The two classes of pyrethroids act on the target site slightly differently. Type 1 compounds prolong the sodium current for milliseconds whereas the type 2 compounds increase the duration of the current for minutes or longer. Therefore, type 1 compounds produce multiple action potentials within the peripheral nerves and interneruons in the central nervous system and type 2 compounds simply reduce electrical excitability through a long-term depolarization of the axon. Due to this variability in the mode of action, the two types of pyrethroids exhibit different signs of intoxication within insects. Type 1 compounds produce hyperexcitability and convulsions within the insect after intoxication and, due to their quick action, a greater knockdown ability when compared to type 2 compounds. Conversely, type 2 compounds produce lethargic actions and ataxia (Bloomquist, 1999). Structure activity experiments have suggested that the more polar molecules are capable
of capable of penetrating the cuticle more efficiently, resulting in rapid knockdown, but quick dissolution from the active site, reducing toxicity (Salgado et al., 1983). It is interesting to note that both DDT and type I pyrethroids display a negative temperature coefficient of toxicity, meaning they are more toxic at low temperatures, whereas type 2 pyrethroids display a positive temperature coefficient (Yu, 2008). Figure 1-2 depicts commonly used pyrethroids for vector control.

One of the oldest groups of commercial insecticides are chloride channel antagonists such as dieldrin, lindane and fipronil (Bloomquist, 1998). Although the majority of these compounds have now been banned from commercial use, lindane and endosulfan are still used in a variety of circumstances due to their inherently higher biodegradability. These inhibitors possess an antagonistic effect on the inhibitory neurotransmitter found within the CNS, gamma-aminobutyric acid (GABA). This antagonism results in hyperexcitability and convulsions, due to the prevention of synaptic inhibition through blocking GABA-mediated Cl⁻ channel activation. Many of these compounds possess high mammalian toxicity, which has led to subsequent banning of many insecticides within this class. However, recent chemical synthesis has produced a novel GABA antagonist, fipronil, which has high selectivity toward insects over mammals and is often used in veterinary medicine (Bloomquist, 1999).

1.6 Insecticide Target Sites and Structural Biology of Acetylcholinesterase

Acetylcholinesterase is a well-validated and highly useful target for insecticides. As previously mentioned, AChE is critical for sustaining life within insects and mammals due to its principal role in nerve signal propagation. It serves to hydrolyze ACh within cholinergic synapses and inhibition of this process will lead to repeated nerve stimulation, tremors, and eventual death of the insect. Although many anticholinesterases have high insect toxicities, their mode of action typically allows for minimal selectivity among commercially vailable
compounds. Both mammalian and insect AChE possesses a serine residue at the catalytic site within the AChE gorge which results in poor selectivity and thereby limits the use of many AChE inhibitors (Pang, 2009). Thus, development of anticholinesterases with a novel mode of action is of critical importance for control of mosquito borne diseases.

The development of a three-dimensional (3-D) structural model of AChE from the electric eel, *Torpedo californica* (*Tc*AChE), has provided an understanding of the structure-function relationships between inhibitors and AChE (Sussman et al., 1991). Moreover, we are now capable of utilizing crystal structures of numerous AChE proteins to perform *in silico* ligand docking of chemical libraries. The *An. gambiae* acetylcholinesterase gorge is approximately 20 Å deep and is comprised of a peripheral site at the mouth of the gorge, a narrowed, ‘bottleneck’ section halfway down the gorge, and a catalytic acyl site at the bottom of the gorge (Sussman et al., 1991). Functionally important residues are shown in figure 1-3. The catalytic acyl site is composed of the catalytic triad (His-441, Glu-325, and Ser-200 (Ag numbering). The π orbitals of tryptophan 84 facilitate binding of the trimethylammonium group of acetylcholine and serine 200 (Ag numbering) functions to quickly bind the acetyl group of the substrate during catalytic hydrolysis (Szegletes et al., 1999). During the carbamylation reaction between the substrate and the CAS, the oxyanion hole is thought to stabilize the tetrahedral intermediate of the reaction (Szegletes et al., 1999). The anionic site is thought to bind ACh at the quaternary ammonium group through π-cation or π-π interactions. The peripheral site of the acetylcholinesterase gorge includes cysteine-286 (absent in humans), aspartate-72 and tryptophan-279 (Szegletes et al., 1999; Ferrari et al., 2001; Pang, 2006), and has a unique action during substrate catalysis. It has been suggested that the peripheral site contributes to catalytic efficiency by the transient binding of ACh as it migrates toward the catalytic acyl site. Results discussed in Ferrari et al (2001)
provided a potential explanation of the role for the ligand binding to the peripheral site. It was proposed that the binding of the ligand to the peripheral site initiated a conformational change within the AChE protein structure, which is then allosterically transmitted to the acyl site to facilitate catalysis (Ferrari et al., 2001).

1.7 Insecticide Resistance Mechanisms

Insecticide resistance has become a major problem for effective mosquito control due to a continuous selection for resistance to nearly all deployed insecticides (Pasteur and Raymond, 1996). DDT was first utilized for mosquito control in 1946 and resistance to the insecticide was reported in *Ae. triataeniorhynchus* and *Ae. sollicitans* in 1947 (Brown, 1986). In 1992, there were more than 100 mosquito species that were considered to be resistant to one or more insecticide classes (WHO, 1992).

The biochemistry of insecticide resistance is important to understand when attempting to mitigate resistance through the manipulation of compound chemistries or when performing surveys to understand resistance patterns within an area. There are two primary methods for the production of insecticide resistance: increased concentrations of metabolic enzymes and/or target site insensitivities. Glutathione S-transferases (GST), esterases, and monooxygenases are three major enzyme groups within insects that are believed to be responsible for metabolically based resistance to organochlorines, organophosphates, carbamates, and pyrethroids (Hemingway and Ranson, 2000). Also, non-silent point mutations yield target site insensitivities and are therefore the primary method for producing target site resistance.

Esterases (synonymous with carboxylesterases) are a group of enzymes that hydrolyze carboxylic esters (Walker, 1993; Hemingway and Karunaratne, 1998). The carboxylesterase-based mechanism for resistance has been found within numerous medically important species and is considered the principal mechanism for OP resistance and the secondary mechanism for
CB resistance within mosquitoes, including *An. gambiae* (Hemingway and Karunaratne, 1998; McCarroll et al., 2000). There are two classes of esterases, A and B. Some A esterases can hydrolyze OP insecticides through the use of an acylated cysteine in the active site and are therefore commonly termed phosphoric triester hydrolases (EC: 3.1.8) (Aldridge, 1953; Reiner et al, 1993; Walker, 1993). B esterases have a serine residue within its active site and are classified as serine hydrolase enzymes. The mechanism for insecticide resistance is thought to be due to sequestration rather than metabolizing the xenobiotic, which is seen in various other metabolic based resistances (Kadous et al., 1983). When AChE-inhibiting xenobiotics are substrates for these enzymes, the acylated enzyme is formed quickly but the deacylation step is slow and becomes the rate limiting step. Therefore, the increased presence of carboxylesterases produces resistance due to rapid sequestration before the insecticides reach the target enzyme.

GSTs are commonly found in aerobic organisms and are dimeric multifunctional enzymes that metabolize a large range of xenobiotics to confer insecticide resistance to a variety of insecticide classes (Ranson et al., 2001; Prapanthara et al., 1996). Resistance due to elevated levels of GST activity was initially identified in OP resistance and it is now considered to be a mechanism of metabolic resistance within many insect species, including *An. gambiae* (Hayes and Wolf, 1988). Detoxification of OP insecticides occurs via an $O$-dealkylation (glutathione conjugation with the alkyl group of the insecticide) or an $O$-dearylation (glutathione conjugation with the leaving group) reaction (Oppenorth et al., 1979; Chiang and Sun, 1993).

Cytochrome P450 monooxygenases are a group of enzymes found in the majority of animals, including insects. Similar to GSTs, these enzymes are involved in the metabolism of xenobiotics and are associated with pyrethroid resistance in numerous mosquito vectors. The P450 monooxygenases metabolize a large majority of insecticides, usually for detoxification
purposes, but are occasionally involved in bioactivation reactions, as seen with OPs. The catalytic action of P450 enzymes is through the donation of an oxygen molecule into the substrate via uptake of electrons from NADPH (Hemingway et al., 1985; Vulule et al., 1994; Brogdon et al., 1997).

Target-site resistance is the second most common method of insecticide resistance and is produced through point mutations (Hemingway and Ranson, 2000). For resistance to occur, the amino acid change must yield a reduction in affinity or efficacy for the insecticide without reducing the functionality of the target site too extensively. Although the point mutations confer resistance towards insecticides, there is a negative fitness cost to the individual in the absence of the insecticide and therefore, the fitness cost has large implications toward the persistence of the resistance within the field (Hemingway and Ranson, 2000).

Prior to discussion of AChE point mutations that confer resistance, it is important to understand the genes that correspond to AChE. Two primary genes have been discovered which encode AChE, *ace-1* and *ace-2* (Weill et al., 2004). Published studies have reported that AChE1 is the primary site for OP and CB binding, implying that the *ace-1* gene encodes the primary AChE in many insect species (Chen et al., 2009). Therefore, the insect *ace-1* gene is likely more important in comparison to the *ace-2* gene; however, *Drosophila melanogaster* and *Musca domestica* are two insect species that utilize only the *ace-2* gene for encoding the primary AChE enzyme. Both *ace* genes, *ace-1* and *ace-2*, were found to be present within AgAChE, however it is well established that *ace-1* is the primary gene for encoding the AChE enzyme within AgAChE (Weill et al., 2002). The function of *ace-2* when both *ace* genes are present is unknown (Weill et al., 2002; Weill et al., 2004). A major difference between AChE1 and AChE2 (encoded by *ace-1* and *ace-2* respectively) is a 31 amino acid insertion within the AChE2
sequence. This insertion is absent in vertebrate AChEs and is potentially a characteristic of the
ace-2 gene in diptera (Weill et al., 2002).

Insensitive AChE is a common resistance mechanism to anticholinesterase insecticides in
insects. However, the point mutations have been found to display a range of insensitivities due
to variability in the mutation and variability within the genes encoding AChE (Weill et al.,
2004). Mutations corresponding to AChE insensitivity towards insects that utilize the ace-2 gene
(ie: Drosophila melanogaster, Musca domestica, and Bactrocera oleae) have been well
described, but the different behavior of the ace-1 gene has hindered the process for insects that
do not utilize ace-2 (Malcom et al., 1998). High levels of AChE insensitivities have been
documented for mosquito vectors including: An. gambiae (Weill et al, 2003), An. albimanus
(Ayad and Georgiou, 1975), and Cx. pipiens (Bourguet et al., 1996). High levels of AChE
resistance within Cx. pipiens and An. gambiae corresponds with the same glycine to serine
substitution (known as G119S through Torpedo nomenclature), and results from a single point
mutation of GGC to AGC in the ace-1 gene (Weill et al., 2003). MACE (Modified
AcetylCholinEsterase) is used to describe insensitive AChE enzyme due to a point mutation that
yields high resistance ratios toward dimethylcarbamates, such as pirimicarb (Foster et al., 2003).

1.8 Insecticide Resistance Patterns Observed in Arthropod Disease Vectors

The emergence of widespread insecticide resistance is threatening disease control efforts
by allowing a reemergence of insect vector populations. In An. gambiae populations, resistance
to OPs emerged 14 years after their initial deployment and five years after the first uses of
carbamates (Hirshorn, 1993). The majority of insecticide resistance within populations of An.
gambiae is to pyrethroids by means of an increased frequency of kdr (altered sodium channel)
and increased levels of elevated P450 monooxygenases (Vulule et al., 1999; Ranson et al.,
2000b; Stump et al., 2004). Anticholinesterase resistance is not yet widespread in An. Gambiae
mosquitoes from Kenya, as the majority of exposure to carbamates and organophosphates is likely through agricultural uses. However, AChE resistance has been found in Southern Benin within the AKRON strain of *An. gambiae*. The AKRON strain possesses upregulation of metabolic genes corresponding to cytochrome P450’s (CYP6P3: 12.4 fold, CYP6M2: 2.5 fold, and CYP325D2: 5.1 fold) and to elevated GST levels (GSTD1-6: 2.7 fold, GSTD11: 2.1 fold) (Djouaka et al., 2008). In conjunction with increased metabolic activity, PCR analysis has shown the AKRON strain to possess a *kdr* mutation (*L1014F*) and an insensitive *ace-1* mutation that confer resistance to pyrethroids/DDT and carbamates, respectively (McAllister and Adams, 2010; Yadouleton et al., 2009). Resistance ratios towards carbamates (e.g. propoxur) have been shown to be greater than 5,000-fold when compared to the susceptible G3 strain (McAllister and Adams, 2010).

Other arthropods are also capable of exhibiting high levels of insecticide resistance that reduce the effectiveness of control programs. The cattle tick, *Bm*, is a prime example of this as resistant ticks continuously cross the Mexican border and enter the United States, a country that has eradicated *Bm* populations. OP and pyrethroid resistance has been attributed to both metabolic and target site insensitivities, with the later being the primary mechanism for OP resistance (Li et al., 2003; Schuntner et al., 1968; Bull and Ahrens, 1988; Morgan et al., 2009). However, isolating the mutated sequence of insensitive *BmAChE* has proven to be difficult and the situation is convoluted due to the presence of multiple genes encoding *BmAChE* (Temeyer et al., 2010). Temeyer et al. (2010) expressed three acetylcholinesterases from OP resistant and susceptible strains of *Bm* and showed different alleles existed between the two strains. Although future research is needed to determine the roles of each gene, *in vivo*, it appears that OP-insensitivity is multigenic (Baxter and Barker, 1998; Temeyer et al., 2010) or occurs from post-
translational modification of BmAChE1, as shown in *Drosophila melanogaster* (Mutero and Fournier, 1992).

Insecticide resistance in phlebotomine sandfly populations is also a major issue that must be considered when determining control methods or designing novel inhibitors. There have been recent reports of insecticide resistance in sandfly populations (Alexander and Maroli, 2003) and evidence for AChE resistance through target site insensitivities (Surendran et al., 2005). The reports documenting the occurrence of MACE within sandflies were from the countries of Sri Lanka and Sudan, two countries participating in anti-malarial campaigns. It is reasonable to suggest the anticholinesterase resistance is due to widespread use of organophosphates during this time.

Insecticide resistance must be understood and combated to continue controlling arthropod disease vectors. An understanding of the resistance patterns can assist researchers in the development of novel inhibitors and allow the use of additional control measures, such as augmenting the chemical control program with insect repellents for personal protection.

**1.9 Historical and Current Uses of Repellents for Insect Vector Control**

The use of insect repellents likely began thousands of years ago with the use of natural products from plants and insects, primarily utilizing the benzoquinones present in these tissues (Baker, 1996). Historical documentation provides insight into the use of natural repellents, such as castor oil, by the Egyptians, Romans, and Native Americans for the control of flying insects (Romi et al., 2001; Charlwood, 2003).

Although natural repellents do provide a small degree of personal protection from insects, synthetic insect repellents have become a highly effective method of personal protection from biting insects and have functioned to enhance the effectiveness of vector control programs that utilize synthetic insecticides. In the early 1930’s, militaries around the world conducted
extensive research to design a repellent adequate enough to protect troops on deployment. The first wave of military repellents contained essential oils derived from plants, such as citronella and camphor (Covell, 1943). However, use of these repellents was short lived as the repellent properties were minimal and the duration of action of the repellent was short. During this time, U.S. troops were suffering enormous bouts of malaria in the various theaters of war during the 1940’s that lead to 302 deaths (Mowry, 1963) and 12 million lost duty days (Bunn et al., 1955). Along with malaria, chigger-borne scrub typhus became a large issue with the advent of jungle warfare in the Pacific War. Due to the rise of disease amongst our wartime troops, the United States Department of Agriculture (USDA) was requested in 1942 by the Department of Defense to search for new insecticides and repellents to control mosquitoes and chiggers (Whayne, 1955).

In 1952, a breakthrough in the science of repellents occurred with the discovery of \(N,N\)-diethyl-3-methylbenzamide, or DEET, by the United States Army (McCabe et al., 1954). DEET has been on the market for over 50 years and remains the most effective insect repellent in use today. DEET is a broad spectrum repellent that is highly effective against all mosquitoes (Gupta and Rutledge, 1991; Trongtokit et al., 2004; Curtis et al., 1987), sand flies (Alexander et al., 1995a), black flies (Robert et al., 1992), chiggers (Frances, 1994), ticks (Solberg et al., 1995), bedbugs (Kumar et al, 1995), and fleas (Mehr et al., 1984).

An estimated 200 million people around the world use DEET-containing products each year (U.S. EPA, 1980). Due to the high frequency of use, there have been extensive debates on the safety of DEET after a variety of reports indicate a potential linkage of DEET to seizures and encephalopathy (Roland et al., 1985; Osimitz and Murphy, 1997; Briassoulis et al., 2001). There has also been recent documentation that DEET is neurotoxic and is synergized by neurotoxic insecticides (Osimitz and Grouthaus, 1995; Abou-Doina et al., 1996). Furthermore, mode of
action studies suggests DEET is an acetylcholinesterase inhibitor (Corbel et al., 2009). While a significant amount of literature exists on the effects of DEET to human toxicity within a laboratory setting, there have been minimal clinical reports of adverse effects directly linked to the use of DEET, even though billions of doses are applied annually. It is also interesting to note that the frequency of observed seizures occurring after use of DEET containing products, when analyzed in the context of the number of human applications, any linkage is voided based on the increased probability of an association by chance (Koren et al., 2003).

Though many years of research has been performed on numerous aspects of DEET action, it is surprising that a debate remains as to the repellency mode of action and molecular targets of DEET. One argument suggests DEET is a blocker of olfactory sensory neurons to attractants, such as carbon dioxide and 1-octen-3-ol (Ditzen et al., 2008; Dogan et al., 1999; Davis and Sokolove, 1976). Other research has suggested that insects detect DEET through olfactory mechanisms that elicit avoidance behavior (Syed and Leal, 2008; Carroll et al., 2005). The experimental evidence for both mechanisms potentially indicates that DEET does not affect one specific molecular target, and can therefore provide repellency through multiple modes of action. An understanding of the molecular mode of action for both, repellency and toxicity is essential for the development of novel compounds and for continued control of vector borne diseases.

1.10 Development of New Anticholinesterase Inhibitors

The current problems associated with insecticide use, such as increased insecticide resistance, have resulted in a need for exploring new chemicals with alternate target sites to sustain the vital role of insecticides in vector borne disease control. However, newer tools in insecticide design can assist us in modifying the existing insecticide compounds for known target sites, of which AChE is one. The three-dimensional structure of AChE from Torpedo californica
(TcAChE) is available and provides insights for studying structure–function relationships of many inhibitors (Sussman et al., 1991). Crystal structures of other AChE proteins are available, which assisted molecular modeling efforts and the synthesis of appropriate libraries of anticholinesterase compounds (Bartolucci et al., 2001; Bourne et al., 2004). The AChE enzyme of *Drosophila melanogaster* has been crystallized and provides structural insights for other insect AChEs. It has shown that the insect AChE gorge is narrower than the previously crystallized structure of *Torpedo californica* and smaller in gorge volume (Harel et al., 2000). This crystal structure can be utilized to determine the structure and function of other insect AChE gorges through comparative molecular modeling, bearing in mind that there are two ace genes in insects and the *Drosophila* crystal structure belongs to ace-2.

Molecular models suggest that the peripheral and active sites of AgAChE and human AChE (hAChE) consist of differing and unique amino acids, which can assist in the design of a selective carbamate (Pang, 2006; Carlier et al., 2008). Effects of ligand binding to the catalytic triad due to the interactions between three of the peripheral site residues and the formation of the ‘bottleneck’ in the insect gorge has been studied through site directed mutagenesis (Mutunga, 2011). Through utilization of these models, design of selective compounds that are capable of interacting with key amino acids is possible. Figure 1-4 shows experimental compounds that demonstrated high potency and selectivity to AgAChE (Carlier et al., 2008) and will be used in this study. Figure 1-5 displays PRC 521, a non-selective experimental carbamate. Table 1-1 displays the potency and mosquito selectivity of our novel inhibitors toward AgAChE and human AChE.

### 1.11 High-Throughput Screening of Insecticides

Researchers have attempted to standardize the HTS process (ie: solvents for inhibitors) in order to increase production and decrease variability. Dimethyl sulfoxide (DMSO) has favorable
characteristics (good dissolving ability, low chemical reactivity, etc.) and therefore has been chosen for the standard vehicle during HTS (Tjernberg et al., 2006). Because DMSO has become the standard solvent for drug discovery, there has been a large amount of research on the storage of compounds within DMSO stock solutions. Many of these articles discuss how prolonged storage can potentially cause compound instability, decreased potency through freeze/thaw cycles, and the effect of water absorption toward compounds (Cheng et al. 2003; Kozikowski et al., 2003). While this knowledge is important, there has been little documentation on the critical aspect of the effects of DMSO on protein function during *in vitro* HTS. Research has shown DMSO can act as a stabilizer (Rajendran et al., 1995), denaturant (Jacobson and Turner, 1980; Fujita et al., 1982; Bhattacharjya and Balaram, 1997; Kovrigin and Potekhin, 1997), inhibitor (Perlman and Wolff, 1968; Johannesson et al., 1997; Kleifield et al., 2000), or an activator in various systems (Rammler, 1967). However, these experiments were performed at DMSO concentrations (10% - 70%) that exceed the 0.1% (v/v) to 5% (v/v) typically used for *in vitro* applications, such as HTS (Tjernberg et al., 2006). It is vital to understand the interaction between solvent and enzymes when attempting to develop a selective insecticide. Without this knowledge, advantageous potency, efficacy, or selectivity can be overlooked due to solvent-dependent effects during *in vitro* assays.

### 1.12 Objectives of This Study

The dissertation describes three research objectives:

**Objective 1:** The first objective was to perform experiments to investigate the biochemical mechanism of a solvent-dependent antagonism of AChE inhibition by mosquito-selective carbamates within AgAChE. An understanding of solvent-enzyme interactions during *in vitro* assays is critical for the proper screening of compounds against AChE. Details of this study are presented in Chapter 2.
Objective 2: The second objective of this dissertation work was to characterize the inhibitor profile of recombinant acetylcholinesterases from two arthropods, the cattle tick (*Boophilus microplus*) and the sandfly (*Phlebotomus papatasi*), and was compared to human and bovine AChE, in order to identify divergent pharmacology that might lead to selective inhibitors. Specifically, known AChE inhibitors, experimental carbamate inhibitors, and a tacrine dimer series were used as structural probes to determine AChE protein biology. Details of the AChE inhibitor profile and the potential leads for future insecticide design are presented in Chapter 3.

Objective 3: The third objective of my dissertation research investigated the mode of action of biting insect repellents, such as DEET, upon ion channels and enzymatic systems. A thorough understanding of DEET neurotoxicity is vital for its continued use as a repellent and can provide information on the design of future insect repellents. Complete details of DEET neurotoxicity through toxicokinetic assays, biochemical assays, and electrophysiological recordings are presented in Chapter 4.

Objective 4: The fourth objective of my dissertation was to determine the activity of our novel carbamate inhibitors to nuisance biting mosquito vectors and agricultural pests. Accounting for mosquito resistance toward insecticides is vital when developing mosquitocides for disease control. The onset of insecticide resistance can be dramatically delayed through the development of chemicals with low toxicity to agricultural pests due to limited selection pressure within breeding sites. The undocumented insect selectivity of our novel carbamates is presented in Chapter 5.
Figure 1-1. Two commonly used carbamates (A, B) and organophosphates (C, D) for controlling disease carrying mosquito vectors and other insect pests. Coumaphos oxon is shown as it is the active form of the chemical.

Figure 1-2. Displays structures of two synthetic pyrethroids (A, type 1 and B, type 2) and the structure of DDT (C).

A) Permethrin

B) Deltamethrin

C) Dichlorodiphenyltrichloroethane (DDT)
Figure 1-3. Diagram of AgAChE gorge showing some of the relevant amino acid residues (TcAChE numbering) at the peripheral (W279, C286, W431), anionic site (W84), and catalytic acyl sites (S200).
Figure 1-4. Highly selective and species-sensitive carbamate molecules designed for use on *Anopheles gambiae*, and referred to in this study. IUPAC names of the experimental compounds are: 3-(t-butyl)phenyl methylcarbamate (Terbam, Knockbal, TBPMC, PRC 331), 3-(ethylidimethylsilyl)phenyl methylcarbamate (PRC 337), 3-(trimethylsilyl) phenyl methylcarbamate (PRC 387), 3-(ethylidimethylsilyl)phenyl methylcarbamate (PRC 388), 2-(2methylbutylthio)phenyl methylcarbamate (PRC 407), 2-(ethylbutylthio)phenyl methylcarbamate (PRC 408), and 2-(2-ethylbutoxy)phenyl methylcarbamate (PRC 421). In Chapter 3 of the dissertation, these compounds are numbered with bold Arabic numerals instead of PRC designations.
Figure 1-5. Non-selective experimental carbamate, PRC 521. IUPAC name: 3-\(\text{(sec-butyl)}\)phenyl methylcarbamate.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>(\text{An. gambiae AChE IC}_{50}, \text{nM;(95}% \text{ CI)})</th>
<th>Hill slope</th>
<th>(r^2)</th>
<th>(\text{Human AChE IC}_{50}, \text{nM;(95}% \text{ CI)})</th>
<th>Hill slope</th>
<th>(r^2)</th>
<th>MS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propoxur</td>
<td>371 (320-421)</td>
<td>0.94</td>
<td>0.99</td>
<td>1710 (1420-2060)</td>
<td>1.31</td>
<td>0.99</td>
<td>5</td>
</tr>
<tr>
<td>PRC 331</td>
<td>124 (2-4)</td>
<td>0.50</td>
<td>0.99</td>
<td>9551 (240-293)</td>
<td>0.98</td>
<td>0.99</td>
<td>88</td>
</tr>
<tr>
<td>PRC 337</td>
<td>6 (117-132)</td>
<td>0.84</td>
<td>0.99</td>
<td>9551 (7695-11850)</td>
<td>0.97</td>
<td>0.98</td>
<td>77</td>
</tr>
<tr>
<td>PRC 387</td>
<td>3 (3-9)</td>
<td>0.71</td>
<td>0.96</td>
<td>532 (375-756)</td>
<td>0.81</td>
<td>0.97</td>
<td>89</td>
</tr>
<tr>
<td>PRC 407</td>
<td>3 (25-36)</td>
<td>0.77</td>
<td>0.99</td>
<td>3543 (3152-3904)</td>
<td>0.95</td>
<td>0.99</td>
<td>118</td>
</tr>
<tr>
<td>PRC 408</td>
<td>276 (2-4)</td>
<td>0.86</td>
<td>0.98</td>
<td>98820 (3182-4134)</td>
<td>1.06</td>
<td>0.99</td>
<td>1204</td>
</tr>
<tr>
<td>PRC 421</td>
<td>9 (245-302)</td>
<td>0.96</td>
<td>0.99</td>
<td>12 (8-16)</td>
<td>1.52</td>
<td>0.98</td>
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</tr>
<tr>
<td>PRC 521</td>
<td>(7-12)</td>
<td>0.59</td>
<td>0.99</td>
<td>(8-16)</td>
<td>0.77</td>
<td>0.99</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*Mosquito Selectivity = \(\text{IC}_{50}\) of Human AChE / \(\text{IC}_{50}\) of mosquito AChE
CHAPTER 2
REDUCED POTENCY OF INSECT-SELECTIVE CARbamates MEDIATED BY ALLOSTERIC DMSO STABILIZATION OF ANOPHILES GAMBIAYE ACETYLCHOLINESTERASE: IMPLICATIONS FOR HIGH THROUGHPUT SCREENING OF INSECTICIDES

Abstract: The increasing prevalence of pyrethroid-resistant mosquitoes within malaria endemic regions has amplified the need for development of effective and selective mosquitocides for use in disease control programs. Towards this end we have explored the selectivity of a series of substituted phenyl methylcarbamates for inhibition of An. gambiae (Ag) acetylcholinesterase (AgAChE) over human AChE (hAChE). Initially, inhibition of these compounds was studied using a fixed ratio of solvent to inhibitor concentration, whereby the final DMSO concentration varied from 0.1% to 0.00000001%. Interestingly, screening of several carbamates in the presence of constant 0.1% DMSO (v/v) indicated reduced inhibition of AgAChE compared to the variable % DMSO method, but no reduction of inhibition was observed with hAChE. For several compounds, this phenomenon resulted in a reduction of the observed hAChE/AgAChE selectivity ratio by at least 10-fold. Commercial carbamates propoxur and bendiocarb displayed no solvent-dependent antagonism of inhibition toward AgAChE or hAChE. The selectivity of novel carbamates and the antagonistic effects observed are potentially explained through amino acid variability within the insect and human AChE proteins, producing varying degrees of flexibility. Molecular modeling suggests the DMSO molecule is capable of reducing the flexibility of the Ag protein and limiting access of the carbamates to the catalytic acyl site of AgAChE. Therefore, it is vital to account for interactions between the solvent and protein/ligand during the development of high-throughput screening methods, especially for these insecticides.

Disclaimer: This study was a continuation of my thesis project (Swale, 2009) performed at Virginia Tech. The thesis was published with single IC\textsubscript{50} values whereas this document contains mean (n=3) IC\textsubscript{50} values in Table 2-1 along with different analysis of inhibition values
and Hill slope values. At the University of Florida, enzyme kinetic (eg. $k_i$, $K_m$, and $V_{max}$) and time course inhibition studies were performed to expound on the previous findings published in Swale (2009). Figures 2-2 and 2-4 were first shown in the thesis and are also included here for completeness.

2.1 Introduction

The need for novel insecticides for disease control is increasing rapidly due to the development of insecticide resistance within mosquito species and the banning of longstanding commercial compounds (Zaim and Guillet, 2002). However, it is becoming increasingly difficult to commercialize new insecticides due to cost of production, strict environmental regulations, and increasing regulatory procedures within the development process. To increase the possibility of discovering novel compounds, there has been an ongoing transition from whole organism testing towards in vitro and/or in silico high throughput screens (HTS) for insecticide discovery (Tietjen et al., 2005). The success of HTS is contingent on a number of factors including, but not limited to, quality of the chemical library, the type of assay used, and the solvent effect on proteins during the assay (Tjernberg et al., 2006).

Standardization of the HTS process, including the solvents used, has expedited screening and decreased response variability of chemical libraries (Ridley et al., 1998). Dimethyl sulfoxide (DMSO) is a simple amphipathic molecule and is the primary solvent used for solubilization of chemical libraries for HTS due to its good dissolving ability, low chemical reactivity, and low vapor pressure (Tjernberg et al., 2006). Since DMSO has become the accepted solvent for the screening of chemical libraries, research has been performed on the stability and decreased potency of inhibitors dissolved in DMSO during freeze/thaw cycles, prolonged storage, or due to water absorption (Cheng et al., 2003; Kozikowski et al., 2003). Previous studies have also analyzed the effect DMSO has toward enzyme systems and has been shown to act as a stabilizer.
(Rajendran et al., 1995), denaturant (Kovrigin and Potekhin, 1968; Jacobson and Turner, 1980; Bhattacharjya and Balaram, 1997), inhibitor (Perlman and Wolff, 1968; Johannesson et al., 1997; Kleifield et al., 2000;), or an activator (Rammler, 1967). However, experiments studying the effect of DMSO on proteins have been predominantly performed at DMSO concentrations of 10%-70% (Tjernberg et al., 2006), greatly exceeding the commonly used concentrations of 0.1% - 5% DMSO during HTS (Tjernberg et al., 2006).

Acetylcholinesterase (AChE: EC 3.1.1.7) is a serine hydrolase necessary for breakdown of the neurotransmitter acetylcholine (Ach) in both human and insect central nervous systems (O’Brien, 1967; Radic and Taylor, 2008). Inhibition of this enzyme prevents termination of nerve signalling, producing hyperexcitation, convulsions, and death (O’Brien, 1967). A plethora of commercial insecticides target AChE, but these inhibitors modify a ubiquitous catalytic serine residue, limiting the selectivity of currently available compounds (Gibney et al., 1990; Radic and Taylor, 2008). This poor selectivity against human AChE reduces the utility of anticholinesterases for use in close proximity to humans, and raises concern for their domiciliary use in disease control programs targeting the malaria mosquito, *An. gambiae*. Although sequence identity between the catalytic triad of human and *Ag ace-l* (the gene responsible for encoding *AgAChE*) is high, residue variability within the acyl pocket and peripheral site potentially allow for the development of selective anticholinesterases (Carlier et al., 2008). Work in our laboratory has identified several substituted phenyl methylcarbamates that possess high selectivity (>100-fold) for the malaria vector *AgAChE* over *hAChE* (Carlier et al., 2008).

Experimental carbamates were originally screened with one protocol, and selection of solvent was based upon the chemical evidence of high compound solubility, typically in ethanol or methanol. However, the selectivity ratios observed previously (Carlier et al., 2008) became
un-replicable when our experimental procedures were standardized to have a constant percentage (0.1%, v/v) of dimethyl sulfoxide (DMSO) throughout the experimental concentrations of the inhibitor. This discrepancy in results led to numerous experiments to characterize the mechanisms involved and a potential explanation for the apparent solvent-dependent antagonism of inhibition seen with AgAChE. The objective of this investigation was to determine the effects of DMSO on carbamate inhibition, and whether these solvent effects can mask selectivity of novel insecticides through solvent-dependent antagonism of inhibition during \textit{in vitro} assays. Implications of these studies for the high throughput screening of insecticides are discussed within.

\textbf{2.2 Materials and Methods}

\textbf{2.2.1 Inhibitors, Solvents, and Assay Reagents}

Standard and commercial carbamates, along with their IUPAC names, are given in Figure 2-1. Propoxur (99% purity) and bendiocarb (99% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and experimental carbamates (Figure 2-1) were prepared as described in Carlier et al., 2008 (Carlier et al., 2008). All experimental compounds were purified by column chromatography and/or re-crystallization and are >95% pure by $^1$H NMR analysis. Ellman assay (Ellman et al., 1961) reagents are composed of acetylthiocholine iodide (ATCh)(≥ 99% purity), 5,5’-dithiobis-(2-nitro)benzoic acid (DTNB)(99% purity), and sodium phosphate buffer, all of which were purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular sieve OP type 3Å were purchased from Sigma (St. Louis, MO, USA) and were used to prevent water absorption within the DMSO stock. Fifty beads were added into a 100 mL stock solution. These sieves have a diameter of ~2 mm, a pore size of 3Å, and a water absorbing capacity of ≥ 15%.
2.2.2 Enzymes

Three enzymes were used in this study: adult *An. gambiae* homogenate (wild type G3 strain, cultured in the Department of Entomology and Nematology, Emerging Pathogens Institute, Gainesville, FL, USA), CBL (*AChE* recombinant enzyme from Creative BioLabs, Shirley, NY, USA), and recombinant *hAChE* (lyophilized powder, Sigma C1682, St. Louis, MO, USA). Homogenate enzymes were prepared from groups of ten non-blood fed adult female mosquitoes homogenized in 1 mL of ice-cold sodium phosphate buffer (0.1 M sodium phosphate, pH 7.8,) with an electric motor driven glass tissue homogenizer. The homogenate was centrifuged at 5000 x g using a Sorvall Fresco refrigerated centrifuge at 4° C for 5 minutes. The supernatant was used as the enzyme source for the assay, and all enzyme preparations contained 0.3 % (v/v) Triton X-100 and 1 mg/ml BSA. The CBL enzyme (recombinant *AChE* / *ace* – 1) consisted of the catalytic domain sequence D1 – P540. It was designed and expressed in soluble form using the baculoviral-insect cell expression system by Creative BioLabs (CBL, division of Creative Dynamics Inc., Port Jefferson Station, NY, USA). The expression vector pFASTBac and Sf9 insect cells were used. The full-length precursor protein is Swiss-Prot code ACES_ANOGA with the corresponding numbering is D162-D701. From infection of 1 liter of insect cell culture, soluble r*AgAChE* (ace-1) was expressed and purified up to 90% (0.25 mg yield), first with an anion exchange Q column, followed by a Ni²⁺-NTA gravity column. Prior to use in assay, CBL was diluted 300-fold with phosphate buffered Triton x-100, and *hAChE* was diluted 500-fold with phosphate buffered Triton x-100.

2.2.3 Inhibitor Preparation Protocols

Three protocols were utilized to determine the inherent effects of solvent on IC₅₀ and Hill slopes for AChE inhibition. Protocol A was 100-fold dilution of a 0.1 M stock solution (DMSO) of inhibitor, suspended into phosphate buffer at pH 7.8. Serial dilutions in 10-fold steps were
then performed in buffer providing a constant ratio of inhibitor to solvent. Final inhibitor concentration range was $10^{-4}$ M to $10^{-11}$ M, with the DMSO content being 0.1% (v/v) at the highest inhibitor concentration used, and declining in 10-fold steps. Protocol B was identical to protocol A with the only exception being the starting stock concentration is 10 mM versus 0.1 M. Protocol C entailed dilution of 0.1 M stock solution of inhibitor dissolved with DMSO. Ten-fold serial dilutions of the stock inhibitor was then performed in solvent to give a range of DMSO stock solutions that were then further diluted into 0.1 M sodium phosphate buffer. A constant final 0.1% (v/v) DMSO was therefore present throughout the experiment, so that the inhibitor concentration was the only variable in the experiment.

2.2.4 Enzyme Inhibition Assays

The Ellman assay (Ellman et al., 1961) was used with slight modifications from Carlier et al. (2008) to determine the bimolecular rate constant ($k_i$). Enzyme solution (10 µL) was added to each well of the 96-well micro assay plate along with 20 µL of dissolved compound and 150 µL of ice-cold phosphate buffer. The plate was incubated at 25°C for six minutes at one minute intervals. Ellman assay reagents ATCh (0.4 mM, final concentration) and DTNB (0.3 mM, final concentration) were prepared fresh and 20 µL was added to the enzyme to initiate the reaction. Changes in absorbance were recorded by a DYNEX Triad spectrophotometer (DYNEX Technologies, Chantilly, VA, USA) at 405 nm. Samples were analyzed at concentrations bracketing the IC$_{50}$ using a three-minute time point to ensure linearity, and contained 0.1% DMSO (v/v) or $10^{-5}$ % DMSO (v/v). Experiments were performed at $10^{-5}$ % DMSO (v/v) due to previously determined experimental evidence of insignificant influence of the DMSO molecule on the AgAChE protein. Bendiocarb was used in place of propoxur due to higher potency against AgAChE.
2.2.4.1  

**k_i determinations**

The $k_i$ results were analyzed via the two-step method (Bar-On et al., 2002; Copeland, 2005) with the use of GraphPad Prism™ 4.0c (GraphPad Software, San Diego, CA, USA). A linear regression was first performed (x-axis: incubation time; y-axis: $V/V_o$, where $V =$ velocity after incubation time, $T$ and $V_o =$ initial enzyme reaction velocity) on five pre-determined concentrations, based upon the $IC_{50}$ value of the inhibitor. The natural log of the previously determined $V/V_o$ ratio was then plotted against incubation time to determine $k(obs)$ values. The $k(obs)$ values were then plotted against the inhibitor concentration, and analyzed by an additional linear regression. The slope of this regression line corresponds to the $k_i$ of the carbamoylation reaction (Bar-On et al., 2002; Copeland, 2005).

2.2.4.2  

**V_max and K_m determinations**

Methods for $V_{max}$ and $K_m$ determinations were nearly identical as previously explained in section 2.4a. However, seven separate ATCh concentrations were used versus the fixed concentration described above. Experiments were performed with 0.1% DMSO ($v/v$) and no DMSO in the same 96-well microassay plate to allow for simultaneous reading and reduced variability. Buffer was used in place of DMSO in the experiments when no DMSO was used. Change in absorbance was analyzed with identical instrumentation as previously described. Samples were analyzed using the 3.67 min time point to ensure linearity using Prism™ (GraphPad Software, San Diego, CA, USA) with the use of the Michelis-Menten equation to determine $K_m$ and $V_{max}$: $V_o = V_{max} [S] / K_m + [S]$; whereby, $V_o$ and $V_{max}$ are the initial and final enzyme velocity, respectively, and [S] designates the substrate concentration.

2.2.4.3  

**IC_{50} determinations**

Inhibition of AChE by solvent or carbamates was determined using the Ellman assay (Ellman et al., 1961) and was based on the method outlined in Carlier et al., 2008. Identical
instrumentation was used as described in section 2.4a and 2.4b. Enzyme concentrations used were within the linear range, therefore eliminating the need for protein quantification. Solutions were prepared with DMSO diluted into sodium phosphate buffer (pH = 7.8) to create final concentrations ranging from 10% to $10^{-7}$% DMSO. The percent activity remaining for each concentration was determined by the formula: (average optical density per concentration / control optical density) x 100. IC$_{50}$ values for each species were calculated by nonlinear regression from eight inhibitor concentrations using Prism$^\text{TM}$ (GraphPad Software, San Diego, CA, USA). The nonlinear regression equation used was as follows:

$$Y = \text{bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - x)\times\text{Hillslope})});$$

where $x = \text{the logarithm of the concentration}$ and $Y = \text{the response}$. $Y$ starts at the top (normalized 100%) and approaches the bottom (0%) with a sigmoid shape.

The time course experiments were also performed with the use of the Ellman assay (1961). The enzyme was incubated with the inhibitor for a total of 60 minutes, with readings taken at ten-minute intervals, allowing for six data points per assay versus one ten-minute incubation. The results were analyzed identically to those explained above.

The midpoint for the DMSO-dependent antagonism of inhibition of AgAChE was determined with the method for Protocol C. DMSO concentrations were held constant at concentrations ranging from 0.00001% to 1%. CBL enzyme was utilized for this experiment due to consistency of results, when compared to Ag homogenate enzyme. Mean IC$_{50}$ values were calculated from three replicate IC$_{50}$ values determined at each DMSO concentration, as described above.
2.2.5 Statistical Analyses

The IC$_{50}$ and Hill slope values for carbamates run under each protocol were averaged (n=3) and compared by a one-way ANOVA followed by Tukey’s multiple comparison test using GraphPad InStat$^\text{TM}$ (GraphPad Software, San Diego, CA, USA). The DMSO effect on catalytic parameters of the substrate, ATCh, was determined by calculating the percent residual activity using the formula: (concentration OD value / control OD value)$\times$100. The mean (n = 3) percent residual activity was compared with a one-way ANOVA and Tukey’s multiple comparison test (Figure 2-2).

The average (n=3) bimolecular rate constants were statistically analyzed using unpaired t-tests with significance being represented by P < 0.05. The average (n=5) $V_{\text{max}}$ and $K_m$ data were statistically analyzed through the use of a paired t-test (df = 4) with significance represented by P < 0.05. Statistical analyses were performed using InStat$^\text{TM}$ (GraphPad Software, San Diego, CA, USA).

2.2.6 Molecular Homology Models

The homology models of DMSO and AChE were previously published in the M. S. thesis of Daniel Swale (Swale, 2009), but are also shown in this dissertation for completeness. Homology models were generated by Dr. Dawn Wong (Virginia Tech Entomology) and through our collaborations with Drs. Max Totrov and Polo Lam at Molsoft ICM. A computationally refined homology model of AgAChE was generated using Molsoft ICM (Abagyan et al., 1994). The X-ray structure of mouse AChE (mAChE, PDB 1D1N5R) was used as a template for the AgAChE catalytic subunit (D1-P540). The flexible peripheral site loop of DmAChE was used as a template for initial modeling of the corresponding loop region of AgAChE. Loop templates were extracted from the PDB and allowed us to further model the loop of AgAChE, followed by Monte Carlo sampling of the side chains, and energy minimization of the backbone. The RMSD
value of the backbone carbon atoms between the refined AgAChE model and the mAChE template is 0.40 angstroms for 495 matches (Carlier et al., 2008).

2.3 Results

2.3.1 DMSO-Dependent Antagonism of Carbamate Inhibition in AgAChE and hAChE

Protocols A and B resulted in similar IC\textsubscript{50} values for Ag homogenate and AgAChE recombinant enzyme (CBL), as shown in Table 2-1. The largest difference between Protocols A and B for Ag homogenate was a 4-fold increase in IC\textsubscript{50} (PRC 388), and there was a 6-fold increase in IC\textsubscript{50} (PRC 331) on CBL. Table 2-1 also shows that in the presence of constant 0.1% DMSO (Protocol C) resulted in a substantial decrease in inhibition potency of mosquito enzymes for the experimental carbamates (PRC 331, PRC 337, PRC 387, PRC 388, PRC 408, and PRC 421). The increased IC\textsubscript{50} values ranged from a 5-fold increase (PRC 421) to a 43-fold increase (PRC 388) with Ag homogenate. Similarly, less potent IC\textsubscript{50} values were observed for CBL, and ranged from a 3-fold increase (PRC 337) to a 28-fold increase (PRC 331).

However, the commercial carbamates propoxur and bendiocarb displayed little difference between the three protocols. From Protocols A to C, Ag homogenate displayed a mere 1.2 fold increase in IC\textsubscript{50} value for propoxur, and a 1.4 fold increase in IC\textsubscript{50} value for bendiocarb. CBL displayed no significant statistical difference between IC\textsubscript{50} values for propoxur, and bendiocarb displayed only a 1.4 fold increase in IC\textsubscript{50} (Table 2-1). Similarly, a non-selective experimental carbamate (PRC 521) displayed a 1.7 fold increase in IC\textsubscript{50} value from Protocol A to Protocol C on Ag homogenate (Table 2-1), a small increase relative to selective carbamates.

CBL was found to be the most sensitive of the two AgAChE enzymes studied to standard and experimental carbamates, regardless of protocol. Propoxur and bendiocarb were 3.7-fold and 3-fold more potent, respectively, in all protocols to CBL when compared to AgAChE homogenate. In general, experimental carbamates were found to be 2-4 fold more potent to CBL.
when compared to AgAChE homogenate. The largest difference in potency between the two 
AgAChE enzymes was 10-fold and was observed with protocol A and PRC 331. A plot of the 
decreased potency observed with PRC 331 across treatment protocols A-C versus little change in 
potency with propoxur against the CBL enzyme is shown in Figure 2-3.

Contrary to AgAChE patterns of inhibition, hAChE yielded at most a 1.3-fold increase in 
IC$_{50}$ value with experimental carbamates (PRC 337) in the presence of constant 0.1% DMSO 
(Table 2-1). The decreased potency of experimental carbamates toward AgAChE with Protocol 
C, and the lack of effect on hAChE produced drastically lower selectivity ratios (Table 2-2).

Hill slope values of the concentration-response curves were also shown to vary according to solvent concentration, selectivity properties of the inhibitor, and AChE enzyme (Figure 2-2). 
For An. gambiae homogenate, the commercial insecticides displayed little to no statistical 
increase in Hill slope values across protocols A-C. Bendiocarb displayed a 20% increase in Hill 
slope from protocol A to protocol C and a 10% increase was observed for PRC 521 (Table 2-1). 
However, statistically significant increases in Hill slope values were observed with experimental 
carbamates, as PRC 331 displayed a 30% fold increase and PRC 388 displayed a 80% increase 
from protocol A to protocol C (Table 2-1). The CBL enzyme displayed results similar to those 
of the Ag homogenate data, with the Hill slopes of bendiocarb and propoxur slightly increasing 
(1.1 fold), whereas Hill slopes for the selective carbamates increased up to 80% (PRC 331) 
between protocol A to C (Table 2-1). Similar to the inhibition potency data, no statistically 
significant increase in Hill slope values among the three protocols was observed with any class 
of carbamates for human AChE (Table 2-1).

We observed a highly statistically significant (P<0.0001) 3-fold increase in the 
bimolecular rate constant ($k_i$) for PRC 331 inhibiting AgAChE when exposed to constant 0.1%
DMSO, compared to constant 10⁻⁵ % DMSO. However, this effect was not observed with the commercial carbamate, bendiocarb (10% increase, P = 0.31). Similarly, hAChE displayed no significant increase in kᵢ for PRC 331 and bendiocarb. This pattern of results is nearly identical to those found with the IC₅₀ results displayed in section 3.1 (Table 2-3).

2.3.2 Time Course of Inhibition Comparison

Increasing the incubation time of AgAChE and inhibitor decreased the IC₅₀ value for all inhibitors studied over all protocols (Table 2-4). The least and greatest difference between ten and sixty minute incubations was with PRC331 using protocol A (128-fold) and PRC 408 using protocol B (1.7-fold), respectively. Commercial inhibitors had little difference in fold-change between ten and sixty minutes as all protocols ranged from 2.7-fold to 4.9-fold, a difference of 1.8-fold. For commercial carbamates, maximum inhibition was reached with an incubation time of 40 minutes for all three protocols. However, PRC 331 displayed a steady increase in potency with increasing incubation time for protocols A and C. With protocol C, we observed nearly a two-fold increase in inhibition with 60-minutes over 30-minutes. Enzyme inhibition with PRC 408 reached maximum at 40 minutes when using protocol A but didn’t reach maximum inhibition until 50 minutes with protocol C. This finding likely indicates a slower delivery of experimental car bamates to the catalytic site with protocol C when compared to protocol A. However, this is not the case for commercial car bamates as the increase in potency was uniform throughout the three protocols.

2.3.3 Concentration-Dependence of DMSO effects

When the AgAChE enzyme kinetics without DMSO is compared to 0.1% DMSO, the Vₘₐₓ had a small, but statistically significant (P = 0.001) 17% increase in Vₘₐₓ of 0.058 μmoles min⁻¹ to 0.068 μmoles min⁻¹. The Kₘ value also displayed a statistically significant increase (P = 0.01) of 87.4 μM to 133.2 μM when AgAChE was exposed to 0.1% DMSO, a 52% increase. As
shown in Figure 2-2A, Ag homogenate showed inhibition at 10% DMSO, an increase in activity at 1% DMSO, and essentially no change in activity at 0.1% DMSO. CBL enzyme activity followed a similar pattern of DMSO-dependent effects (Figure 2-2A). hAChE displayed strong inhibition at 10% DMSO, about half inhibition at 1% DMSO, and a smaller decrease in activity at 0.1% DMSO (Figure 2-2A). This data was shown in Swale (2009).

PRC 331 and the Ag recombinant enzyme (CBL) was used to determine the midpoint of DMSO-dependent antagonism of inhibition by PRC 331 (Figure 2-2B). The midpoint for DMSO antagonism of IC$_{50}$ value was determined to be 35.2 μM (95% CI: 19.5 μM to 63.8 μM; Hill slope = 1.7; $r^2 = 0.96$), or 0.00025% DMSO. DMSO had substantial antagonistic effects on inhibition at 0.128 M (1% DMSO) to 128 μM (10$^{-3}$ % DMSO), and assays performed at 0.1% DMSO (v/v) contain 12.8 mM DMSO (Figure 2-2B), where the effect was maximal. This data was shown in Swale (2009).

2.4 Discussion

Within HTS screens, DMSO concentrations usually range from 0.1% (v/v) to 5% (v/v), depending on the solubility, required inhibitor concentration range, assay type, and sensitivity of the enzyme to DMSO (Tjernberg et al., 2006). Many researchers perform in vitro assays or HTS screens at solvent concentrations that do not affect the enzyme through denaturation or inhibition (Tjernberg et al., 2006). However, these amounts of DMSO may be problematic for some effects, as we demonstrated a solvent-dependent antagonism of AgAChE inhibition at levels (0.1% DMSO), reflected in higher values for both IC$_{50}$ and $k_i$. However, we observed little to no statistically significant decrease of inhibition for hAChE. The increase in AgAChE IC$_{50}$s and lack of effect on hAChE IC$_{50}$s reduced the previously determined selectivity ratios of the experimental carbamates (Carlier et al., 2008). This apparent reduction of selectivity due to
solvent interactions has implications for high-throughput screening of chemical libraries, as it alludes to the possibility of excluding from further development, compounds that are affected by solvent interactions.

Two standard carbamates (propoxur and bendiocarb) were tested on both AgAChE enzymes and resulted in little or no statistical difference for inhibition among the three protocols. Thus, the presence of DMSO has no effect on enzyme inhibition when these two carbamates are used. The structural conformations of these carbamates allow them to react with the catalytic acyl site despite the presence of DMSO. A non-selective experimental carbamate (PRC 521, 3-sec-butyl) also resulted in little (1.7-fold increase from Protocols A to C) statistical difference between the three protocols, indicating that the structural specificity for selective inhibition and its DMSO sensitivity are quite precise, when compared to its tert-butyl analog PRC331 (Table 2-1). Therefore, it is reasonable to suggest that the mechanism mediating selectivity is also involved in the observed effects of DMSO.

While high concentrations of DMSO affected substrate kinetics, DMSO at 0.1% had little to no direct effect on the catalytic activity of the enzymes (Figure 2-2A). Although Km is affected at 0.1% DMSO, inhibition assays are run at 0.5 mM substrate, where the reaction is zero order. These data suggest that the experimental concentration of 0.1% DMSO, which affected carbamate-induced inhibition, has minimal influence on the catalytic activity of the substrate ATCh by enzyme, whether AgAChE or hAChE. Thus, pre-screening the enzyme for DMSO effects under the usual conditions of the Ellman assay would reveal nothing unusual in the behavior of DMSO under typical assay conditions.

Variability in Hill slope values among protocols presents an interesting aspect of DMSO-dependent effects within the catalytic gorge of AgAChE. Hill slope values approach unity for
potent, monovalent inhibitors (Shoichet, 2006; Table 2-1), but increased DMSO concentration increased IC$_{50}$ values and Hill slopes for experimental carbamates progressing from protocol A-C. Variability of Hill slopes between protocols is difficult to rationalize based on an inhibitor solubility mechanism. Incomplete solubility will decrease the concentration of active ingredient rendering it less potent, yet reduced levels of DMSO gave the most potent IC$_{50}$ values. The low Hill slopes observed with protocol A in both AgAChE enzymes gave the appearance of a two-site binding model, indicating that AgAChE possesses multiple enzyme conformers, likely due to enzyme flexibility. The near unity Hill slopes for Protocol C suggest DMSO-mediated allosteric stabilization of the AChE enzyme increases the time spent within a single, lower affinity conformation. Obviously, there is no DMSO present in commercial formulations of anticholinesterase insecticides, so a multiple site/conformational state interaction, in vivo, may actually occur, at least in the case of AgAchE.

Comparison of hAChE and AgAChE sequences show several differences, primarily within the acyl pocket and the peripheral site, suggesting the potential for development of selective anticholinesterases (Carlier et al., 2008). Molecular homology models have been generated (Figure 2-4) and suggest alternate conformations of W84 and W431 (Ag numbering) within the hydrophobic subpocket of AgAChE, giving the Ag enzyme a high degree of flexibility (Figure 2-4). Presumably, this flexibility would be able to accommodate appropriately branched, ortho or meta-substituted alkanes, via rotation of W84. Alternate conformations of the corresponding residues in hAChE are not easily obtained due to the presence of Y449 (h) versus D441 in AgAChE. Homology models of hAChE reveal the presence of hydrogen bonds between Y449 and the indole nitrogens of W86 and W439 (human numbering), and are shown in Figure 2-4. This interaction prevents flexibility within the hydrophobic subpocket of hAChE, rendering
the gorge less accommodating to branched alkane carbamates. Sequence comparisons reveal D441 of AgAChE is analogous to Y449 of the human. Due to the smaller size of D441 when compared to Y449, it is probable to suggest D441 is too distal to produce hydrogen bonds with W84 and W431, as seen in hAChE. The inability to form hydrogen bonds between D441 and W84 and W431 would provide flexibility within the hydrophobic subpocket of AgAChE, potentially producing the high selectivity ratios of AgAChE over hAChE.

To conclude, the unexpected antagonism of inhibition by DMSO described in this research is potentially explained through the flexibility of W84 and W431 (Ag numbering). The size and molecular orientation of DMSO allows for an interaction between a DMSO molecule and the indole nitrogens of W84 and W431 that produces hydrogen bonds with D441 within AgAChE (Figure 2-4). Hydrogen bonding of these substituents produces an AgAChE enzyme structure similar to that of hAChE and results in a loss of flexibility of the AgAChE hydrophobic subpocket, producing a less accommodating enzyme for the selective carbamates. Thus, the stabilization of the AgAChE enzyme through the presence of 0.1% DMSO (v/v) is potentially responsible for the solvent-dependent antagonism of inhibition seen with our AgAChE selective carbamates, and the Hill slope data suggest that the inhibition of antagonism is due to allosteric stabilization and not to direct competition with the insecticide for its binding site.
<table>
<thead>
<tr>
<th>Structure</th>
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<th>Structure</th>
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<th>Chemical Name</th>
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<td>Propoxur</td>
<td>2-isopropoxyphenyl methylcarbamate</td>
<td><img src="image" alt="Propoxur" /></td>
<td>PRC 388</td>
<td>3-(ethyldimethylsilyl)phenyl methylcarbamate</td>
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<tr>
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<td>PRC 408</td>
<td>2-((2-ethylbutyl)thio)phenyl methylcarbamate</td>
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<td>3-(tert-butyl)phenyl methylcarbamate</td>
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<td>PRC 421</td>
<td>2-(2-ethylbutoxy)phenyl methylcarbamate</td>
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<tr>
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<td>PRC 337</td>
<td>2-((2-methylallyl)thio)phenyl methylcarbamate</td>
<td><img src="image" alt="PRC 521" /></td>
<td>PRC 521</td>
<td>3-sec-butylphenyl methylcarbamate</td>
</tr>
</tbody>
</table>

Figure 2-1. Structure name and chemical name of standard and experimental carbamate insecticides utilized in this study.
Table 2-1. IC$_{50}$ values for An. gambiae homogenate, CBL, and human AChE enzymes exposed to DMSO. Numbers represent mean (n=3) IC$_{50}$ values followed by 95% confidence limits in parentheses. Lowercase letters after 95% CI values represent statistical significance for IC$_{50}$ values among protocols A-C. Uppercase letters after the SEM represent statistical significance for Hill slope values among protocols A-C. Values for each inhibitor not labeled by the same letter represent statistical significance across treatment protocols A-C at P < 0.05.

<table>
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<th>Inhibitor</th>
<th>IC$_{50}$, nM; (95% CI)</th>
<th>Hill Slope (SEM)</th>
<th>Protocol A</th>
<th>IC$_{50}$, nM; (95% CI)</th>
<th>Hill Slope (SEM)</th>
<th>Protocol B</th>
<th>IC$_{50}$, nM; (95% CI)</th>
<th>Hill Slope (SEM)</th>
<th>Protocol C</th>
<th>IC$_{50}$ Ratio (C/A)</th>
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<td>150 (139-161)a</td>
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<td>172 (80-264)a</td>
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<td>33 (29-37)b</td>
<td>0.81 (0.02)B</td>
<td>104 (80-128)c</td>
<td>0.91 (0.02)C</td>
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<td>0.8 (0.02)B</td>
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<td>108 (96 – 118)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>5127 (4871-5383)a</td>
<td>0.97 (0.05)A</td>
<td>1.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRC 421</td>
<td>107,000 (96,540-118,600)a</td>
<td>0.84 (0.02)A</td>
<td>130,800 (32,460-326,000)a</td>
<td>0.77 (0.03)A</td>
<td>112,600 (72,489-152,711)a</td>
<td>1.1 (0.07)B</td>
<td>1.05</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2-2. Representation of reduced mosquito selectivity due to increased IC\(_{50}\) within AgAChE under the presence of constant 0.1% DMSO (v/v), Protocol C. IC\(_{50}\) values are expressed as mean (n=3).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>AgAChE (^a) IC(_{50}), nM; (95% CI)</th>
<th>Human AChE (^a) IC(_{50}), nM; (95% CI)</th>
<th>MS*</th>
<th>AgAChE (^b) IC(_{50}), nM; (95% CI)</th>
<th>Human AChE (^b) IC(_{50}), nM; (95% CI)</th>
<th>MS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propoxur</td>
<td>450 (423-479)</td>
<td>1524 (1250-1850)</td>
<td>3.4</td>
<td>445 (267-623)</td>
<td>1442 (2430-3131)</td>
<td>3.2</td>
</tr>
<tr>
<td>PRC 331</td>
<td>10 (8-13)</td>
<td>250 (208-301)</td>
<td>25</td>
<td>104 (80-128)</td>
<td>233 (154-311)</td>
<td>2.2</td>
</tr>
<tr>
<td>PRC 337</td>
<td>156 (121-184)</td>
<td>6126 (5437-6902)</td>
<td>39</td>
<td>476 (356-596)</td>
<td>8035 (7743-8327)</td>
<td>16</td>
</tr>
<tr>
<td>PRC 388</td>
<td>4 (2-7)</td>
<td>427 (389-468)</td>
<td>106</td>
<td>221 (116-325)</td>
<td>451 (431-470)</td>
<td>2</td>
</tr>
<tr>
<td>PRC 408</td>
<td>12 (8-16)</td>
<td>5064 (4649-5518)</td>
<td>422</td>
<td>106 (85-128)</td>
<td>5127 (4871-5383)</td>
<td>48</td>
</tr>
<tr>
<td>PRC 421</td>
<td>79 (51-112)</td>
<td>107000 (96540-118600)</td>
<td>1354</td>
<td>431 (24-607)</td>
<td>112600 (72489-152711)</td>
<td>261</td>
</tr>
</tbody>
</table>

*Mosquito Selectivity (MS) = IC\(_{50}\) of Human AChE / IC\(_{50}\) of mosquito AChE

\(^a\) Data set 1 was performed by preparing inhibitors following Protocol A

\(^b\) Data set 2 was performed using Protocol C with constant 0.1% DMSO as a solvent.
Table 2-3. Average (n=3) bimolecular rate constant determinations for PRC 331 and bendiocarb on AgAChE and hAChE. Values are $k_i$ (95% CI) *Denotes statistical significance at $P < 0.05$. $k_i$ units = mM$^{-1}$/min$^{-1}$

<table>
<thead>
<tr>
<th></th>
<th>AgAChE homogenate</th>
<th></th>
<th>hAChE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1% DMSO</td>
<td>$10^{-5}$% DMSO</td>
<td>P Value</td>
<td>0.1% DMSO</td>
</tr>
<tr>
<td>Bendiocarb</td>
<td>975 (559-1389)</td>
<td>1116 (796-1436)</td>
<td>0.31</td>
<td>114 (52-175)</td>
</tr>
<tr>
<td>PRC331</td>
<td>1600 (1074-2176)</td>
<td>4587 (4260-4912)</td>
<td>&lt; 0.0001*</td>
<td>541 (330-750)</td>
</tr>
</tbody>
</table>
Table 2-4. Average (n=3) IC\textsubscript{50} values of two commercial and two experimental carbamates over a 60-minute incubation time period with \textit{Anopheles gambiae} homogenate.

<table>
<thead>
<tr>
<th>Propoxur</th>
<th>Protocol A</th>
<th>Protocol B</th>
<th>Protocol C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
</tr>
<tr>
<td>10 min</td>
<td>257 (215-307)</td>
<td>289 (246-328)</td>
<td>312 (278-342)</td>
</tr>
<tr>
<td>20 min</td>
<td>134 (107-177)</td>
<td>172 (143-307)</td>
<td>226 (171-245)</td>
</tr>
<tr>
<td>30 min</td>
<td>92 (82-100)</td>
<td>121 (99-148)</td>
<td>148 (121-162)</td>
</tr>
<tr>
<td>40 min</td>
<td>61 (54-79)</td>
<td>98 (77-112)</td>
<td>104 (88-116)</td>
</tr>
<tr>
<td>50 min</td>
<td>55 (948-64)</td>
<td>93 (72 - 103)</td>
<td>96 (81-109)</td>
</tr>
<tr>
<td>60 min</td>
<td>51 (46-57)</td>
<td>101 (91-112)</td>
<td>99 (84-108)</td>
</tr>
</tbody>
</table>

Bendiocarb

<table>
<thead>
<tr>
<th></th>
<th>Protocol A</th>
<th>Protocol B</th>
<th>Protocol C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
</tr>
<tr>
<td>10 min</td>
<td>108 (101-114)</td>
<td>146 (117-168)</td>
<td>152 (118-195)</td>
</tr>
<tr>
<td>20 min</td>
<td>64 (56-74)</td>
<td>82 (66-109)</td>
<td>90 (78-103)</td>
</tr>
<tr>
<td>30 min</td>
<td>52 (47-58)</td>
<td>68 (58-76)</td>
<td>74 (67-83)</td>
</tr>
<tr>
<td>40 min</td>
<td>38 (35--42)</td>
<td>56 (44-68)</td>
<td>63 (55-72)</td>
</tr>
<tr>
<td>50 min</td>
<td>38 (33-44)</td>
<td>58 (48-69)</td>
<td>58 (52-67)</td>
</tr>
<tr>
<td>60 min</td>
<td>37 (33-42)</td>
<td>53 (47-58)</td>
<td>56 (50-64)</td>
</tr>
</tbody>
</table>

PRC 331

<table>
<thead>
<tr>
<th></th>
<th>Protocol A</th>
<th>Protocol B</th>
<th>Protocol C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
</tr>
<tr>
<td>10 min</td>
<td>10 (7-13)</td>
<td>43 (34-56)</td>
<td>101 (94-109)</td>
</tr>
<tr>
<td>20 min</td>
<td>2 (1-4)</td>
<td>33 (26-43)</td>
<td>83 (76-90)</td>
</tr>
<tr>
<td>30 min</td>
<td>0.5 (0.2-0.7)</td>
<td>37 (29-46)</td>
<td>64 (59-69)</td>
</tr>
<tr>
<td>40 min</td>
<td>0.2 (0.1-0.4)</td>
<td>29 (23-35)</td>
<td>46 (43-48)</td>
</tr>
<tr>
<td>50 min</td>
<td>0.13 (0.06-0.2)</td>
<td>17 (14-20)</td>
<td>44 (38-51)</td>
</tr>
<tr>
<td>60 min</td>
<td>0.08 (0.04-0.1)</td>
<td>26 (17-39)</td>
<td>32 (28-37)</td>
</tr>
</tbody>
</table>

PRC 408

<table>
<thead>
<tr>
<th></th>
<th>Protocol A</th>
<th>Protocol B</th>
<th>Protocol C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
<td>IC\textsubscript{50} (nM; 95% CI)</td>
</tr>
<tr>
<td>10 min</td>
<td>37 (22-51)</td>
<td>59 (43-80)</td>
<td>238 (198-272)</td>
</tr>
<tr>
<td>20 min</td>
<td>24 (11-31)</td>
<td>68 (50-92)</td>
<td>89 (75-105)</td>
</tr>
<tr>
<td>30 min</td>
<td>18 (7-26)</td>
<td>47 (34-64)</td>
<td>91 (74-112)</td>
</tr>
<tr>
<td>40 min</td>
<td>9 (2-17)</td>
<td>51 (43-61)</td>
<td>65 (57-73)</td>
</tr>
<tr>
<td>50 min</td>
<td>11 (4-21)</td>
<td>51 (42-62)</td>
<td>49 (43-56)</td>
</tr>
<tr>
<td>60 min</td>
<td>6 (4-15)</td>
<td>35 (29-42)</td>
<td>49 (43-55)</td>
</tr>
</tbody>
</table>
Figure 2-2. Influence of DMSO on AChE catalytic (A) and inhibitor (B) activity. In A, bars represent average (n = 3) percent activity remaining with error bars representing S.E.M. Bars not labeled by the same letter within each enzyme species represents statistical significance at P < 0.05. For B, EC$_{50}$ of DMSO effect on PRC331-dependent inhibition of recombinant AgAChE (CBL) is shown. Symbols represent mean IC$_{50}$ (n = 3) with S.E.M. bars (Swale, 2009).
Figure 2-3. Comparison of dose-response curves for protocols A-C with PRC 331 and Propoxur with CBL enzyme. Error bars are not shown to prevent cluttering of curves but were found to be less than 5% residual AChE activity.
Figure 2-4. Homology models of hAChE (green ribbons) and AgAChE (red and blue ribbons) and orient from N- to C- terminus. A) Monte Carlo-refined AgAChE homology model that displays selected residues found within AgAChE hydrophobic subpocket and are shown as CPK-colored ball and stick model, with white carbon atoms. Alternate conformation of W431 (Ag) is caused by the binding of a selective ligand and is displayed as black lines. Blue to red ribbon represents N- to C- terminus. B) Overlay of hAChE and AgAChE. Grey dashes represent hydrogen bonding between Y449 (h) and W86/W439 (h). C) Top view of AgAChE with DMSO molecule bound at the ‘allosteric’ hydrophobic subpocket. Bridged hydrogen bonding is shown between the DMSO oxygen and W84 (Ag)/W431 (Ag) with interatomic distances in Å. Bound DMSO molecule, rendered as ball and stick model with pale yellow carbon atoms, is closely flanked by two methionines, M83(Ag) and M438(Ag), and several other side chains (Swale, 2009).
CHAPTER 3
INHIBITOR PROFILE OF RHIPICEPHALUS (BOOPHILUS) MICROPLUS AND PHLEBOTOMUS PAPATASI ACETYLCHOLINESTERASE AND THE IDENTIFICATION OF POTENT N-METHYLCARBAMATES FOR THE CONTROL OF THEIR RESPECTIVE VECTORED DISEASES

Abstract: The cattle tick, *Rhipicephalus (Boophilus) microplus* (*Bm*), and the sand fly, *Phlebotomus papatasi* (*Pp*), are disease vectors to cattle and humans, respectively. The purpose of this study was to characterize the inhibitor profile of acetylcholinesterases from *Bm* (*BmAChE1*) and *Pp* (*PpAChE*) compared to human and bovine AChE, in order to identify divergent pharmacology that might lead to selective inhibitors. Results indicate that *BmAChE1* has low sensitivity (IC\(_{50}\) = 200 µM) toward tacrine, a monovalent CS inhibitor with mid nanomolar blocking potency in all previous species tested. Similarly, a series of *bis*(n)-tacrine dimers, bivalent inhibitors, and peripheral site AChE inhibitors possess poor potency toward *BmAChE*. Molecular homology models suggest the r*BmAChE* enzyme possesses a W384F paralogous substitution near the catalytic site, where the larger tryptophan side chain obstructs the access of larger ligands to the active site. This finding suggests a unique AChE gorge structure in *BmAChE*, a phenomenon that can further support the possibility for design of selective inhibitors. In addition, *BmAChE1* and *PpAChE* have low nanomolar sensitivity to a variety of experimental carbamate anticholinesterases that we originally designed for control of the malaria mosquito, *An. gambiae*. One experimental compound, 2-((2-ethylbutyl)thio)phenyl methylcarbamate, possesses >300-fold selectivity for *BmAChE1* and *PpAChE* over human AChE, and a mouse oral LD\(_{50}\) of >1500 mg/kg, thus providing an excellent new lead for vector control.

3.1 Introduction
Utilization of insecticides for disease vector control remains the most effective component of the integrated vector management approach for the control of vector borne
diseases (Hemingway and Ranson, 2000). The cattle tick, \textit{Rhipicephalus (Boophilus) microplus} (Canestrini; \textit{Bm}), is a potentially deadly pest of cattle since it is a primary vector for babesiosis and anaplasmosis (Graham and Hourrigan, 1977). Economic losses are furthered substantially as normal feeding behavior of tick infestations lead to reduction in milk production and weight gain, as well as overall declines in cattle health (Jonsson et al., 1998). Similarly, the sandfly, \textit{Pp} is a primary vector of numerous zoonotic diseases significant to human health, including leishmaniases and bartonellosis (Desjeux, 2001).

Control programs of these two disease vectors rely largely on the use of insecticides. For control of the cattle tick, the USDA implemented the Cattle Fever Tick Eradication Program (CFTEP), which mandates a quarantine zone, dipping of all imported cattle into organophosphate (eg. coumaphos) solutions, and a 7-14 day quarantine period (Graham and Hourrigan, 1977; Miller et al., 2005; Maroli and Lane, 1991). Similarly, sandfly control is largely based on insecticides through the use of indoor residual spraying with pyrethroids and organophosphates (Morsy et al., 1993), and the use of insecticide treated bednets is a successful and sustainable method for malaria control and has been evaluated for control of \textit{Phlebotomine} sandflies (Maroli and Lane, 1991; Falcao et al., 1991; Mutinga et al., 1992; Morsy et al., 1993; Alexander et al., 1995).

Although these control methods have been effective in reducing \textit{Boophilus} and \textit{Phlebotomus} populations, control has become increasingly difficult due to escalating insecticide resistance among wild populations (Roulston et al., 1968; Jamroz et al., 2000; Miller et al., 2005; Surendran et al., 2005). Organophosphate insecticides, such as coumaphos, are inhibitors of \textit{AChE} (EC 3.1.1.7), a serine hydrolase responsible for terminating nerve signals at the synapses of cholinergic systems within the central nervous system of invertebrates, leading to death (O’Brien, 1967). Organophosphate and pyrethroid resistance has been attributed to both
metabolic and target site mechanisms, with the later being the primary reason for organophosphate resistance (Schuntner et al., 1968; Li et a., 2003; Rosario-Cruz et al., 2004; Surendran er al., 2005; Morgan et al., 2009). Organophosphate-insensitive AChE might provide cross-resistance to insecticides with similar mode of action, such as carbamates. Modification of current compounds can provide increased invertebrate/vertebrate selectivity ratios alongside the potential for development of resistance mitigating compounds.

The three-dimensional crystal structures of AChE from Tc, Dm, and mouse (among others) are available, and provide insights to structure-function relationships for numerous inhibitors (Sussman et al., 1991; Taylor et al., 1991; Bourne et al., 1999; Harel et al., 2000). Pharmacological and structural analyses of AChE have revealed that AChE contains two binding sites for inhibitors: one at the catalytic site (CS) and one near the entrance to the catalytic gorge, the PS. The CS is located about 4 Å from the base of the gorge and consists of S-200, H-440, E-327, and W-84 (Tc numbering), the later serving to bind the trimethylammonium group of acetylcholine (Harel et al., 1993). In turn, the PS is located toward the mouth of the gorge and consists of W279, Y70, D72, H287 (Tc numbering) (Harel et al., 1993; Szegletes et al., 1999; Radic et al., 2006; Mallender et al., 2000). The PS has been shown to briefly bind substrates en route to the CS, thereby increasing catalytic efficiency (Szegletes et al., 1999). Using differences in CS geometry between Ag AChE and human AChE (hAChE), we have developed highly selective anticholinesterase mosquitocides (ie: carbamates) having mosquito selectivity of up to 500-fold (Carlier et al., 2008). Simultaneous occupancy of these two sites through the design of bivalent inhibitors should facilitate the mitigation of AChE target site resistance, since resistance to this type of compound would require the development of multiple mutations in the protein while retaining sufficient functionality.
The objective of the present investigation was two-fold. First, we characterized the inhibitor profile of acetylcholinesterases from rBmAChE1 and pPmAChE compared to human and bovine AChE, in order to identify divergent pharmacology that might lead to selective inhibitors. Secondly, we show evidence of highly potent and selective experimental carbamate inhibitors that can assist in the control of Bm and Pp populations.

3.2 Methods

3.2.1 Inhibitors, Solvents, and Assay Reagents

Propoxur (purity $\geq 99\%$), bendiocarb (purity $\geq 99\%$), edrophonium (purity $\geq 98\%$), eserine (purity $\geq 99\%$), and tacrine (purity $\geq 99\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Experimental carbamates (Figure 3-1) were prepared as described in Carlier et al. (2008). All experimental compounds were purified by column chromatography and/or recrystallization and are $>95\%$ pure by $^1$H NMR analysis. All experimental inhibitors used in this study are shown in Figure 3-1 and the structures of all other inhibitors are found in the literature. Bis(n)-tacrine dimers ($n = 2, 3, 4, 5, 6, 7, 8, 9, 10,$ and $12$ methylenes) were synthesized and purified to $>95\%$ using established procedures (Carlier et al., 1999), and provided by the Carlier laboratory, Department of Chemistry, Virginia Tech, for this work. The inhibitors E2020 ($\geq98\%$ purity), BW284c51 ($\geq98\%$ purity), tubocurarine ($>97\%$ purity), and ethidium (95% purity) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Ellman assay (Ellman et al., 1961) reagents are composed of ATCh ($\geq 99\%$ purity), DTNB (99% purity), and sodium phosphate buffer, all of which were purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular sieve OP type 3Å beads were purchased from Sigma (St. Louis, MO, USA) and were used to prevent water absorption within the DMSO stock.
3.2.2 Molecular Homology Modeling

Molecular homology models were constructed through collaborations with Drs. Maxim Totrov and Polo Lam at Molsoft L.L.C. (LaJolla, CA). Homology models of the BmAChE1 were constructed in ICM (ICM Manual Molsoft 2011). X-ray structure from the Protein Databank (PDB ID 1ACJ – the complex of Torpedo Californica AChE with tacrine) was used as a template. Side-chain refinement was performed in ICM using Biased Probability Monte-Carlo (BPMC) global optimization procedure (Abagyan and Totrov 1994).

3.2.3 Enzyme Preparations

Recombinant enzymes were provided by Drs. Kevin Temeyer and Beto Perez de Leon at the USDA-ARS in Kerrville, TX. Recombinant constructs of R. (B.) microplus BmAChE1 were produced as previously described (Temeyer et al., 2010), except that baculovirus supernatants containing rBmAChE1 were produced in sf21 insect cell culture grown in Gibco® Sf-900™ III SFM (serum-free medium, Life Technologies, Carlsbad, CA). Site-directed mutagenesis was utilized to convert the codon for Trp384 to Phe384 (W384F) in cDNA of BmAChE1 (Deutch #5, wt) previously cloned into the baculoviral transfer plasmid pBlueBac4.5/B5-His-TOPO® (Life Technologies) as previously described (Temeyer et al., submitted). Briefly, 5’-phosphorylated PCR primers BmAChE1-1203U29X (CTTCTTCTTGCAATACTTCTT CGGATTTC) and BmAChE1-1181L22 (GAACCTTCGTTTGCGTTAGAAC) were utilized (25 cycles, 66°C annealing temp, 4 min extension at 72°C) with the Phusion® Site-Directed Mutagenesis Kit (Thermo Fisher Scientific, Pittsburg, PA) to perform targeted mutagenesis following the instructions of the manufacturer. The mutagenized plasmid was transformed into E. coli TOP10 chemically competent cells, sequence verified, and cotransfected with Bac-N-Blue™ DNA into Sf21 insect cells as previously described. Baculovirus cultures were produced in sf21 cells
grown in Gibco Sf-900™ III SFM. Baculoviral DNA was isolated and sequenced from all expression cultures to verify construction and expression of the intended coding sequences.

Six enzymes were utilized in this study: rBmAChE1, mutated rBmAChE1 (W384F), rPpAChE, hAChE, bovine brain homogenate, and Ag AChE homogenate. AgAChE and bovine brain homogenate enzyme was prepared from groups of ten whole non-blood fed adult female mosquitoes or 5 mg (wet weight) of excised bovine brain tissue. Bovine tissue collection was via a local slaughterhouse, and approved by the University of Florida IACUC. Each tissue was homogenized in 1 mL of ice-cold sodium phosphate buffer (0.1 M, pH 7.8) containing 0.3% Triton x-100, with an electric motor driven glass tissue homogenizer. The homogenate was centrifuged at 5000 x g using a Sorvall Fresco refrigerated centrifuge, at 4°C for 5 minutes. The supernatant was used as the enzyme source for the assay. Prior to use in assay, rBmAChE1 and PpAChE were diluted 10x and hAChE was diluted 100x with the aforementioned buffer + Triton mixture.

3.2.4 Enzyme Inhibition Assays

IC₅₀ values were determined using slight modifications from Ellman et al. (1961), as outlined in Hartsel et al. (2012). Briefly, ten µL of enzyme solution was added to each well of a 96-well micro assay plate, along with 20 µL of dissolved compound and 150 µL of ice-cold phosphate buffer. The assay plate was incubated at 25°C for ten minutes. Ellman assay reagents, ATCh (0.4 mM, final conc.) and DTNB (0.3 mM, final conc.), were prepared fresh and 20 µL was added to the enzyme to initiate the reaction. Changes in absorbance were recorded by a DYNEX Triad spectrophotometer (DYNEX Technologies, Chantilly, VA, USA) at 405 nm. Six inhibitor concentrations were used in triplicate to construct concentration-response curves. Inhibitors were prepared using DMSO and contained a final concentration of 0.1% DMSO (v/v).
throughout each inhibitor concentration. Enzyme concentrations used were within the linear range of activity, therefore eliminating the need for protein quantification.

### 3.2.5 Statistical Analyses

Individual IC$_{50}$ values were calculated using nonlinear regression with GraphPad Prism$^\text{TM}$ (GraphPad Software, San Diego, CA, USA). All experiments yielded acceptable Hill slope (>0.8) and $r^2$ ($\geq 0.99$) values. IC$_{50}$ values are expressed as mean of n=3 values. Mean IC$_{50}$ values and 95% confidence limits were determined with GraphPad InStat$^\text{TM}$ (GraphPad Software, San Diego, CA, USA). The mean IC$_{50}$ values were statistically analyzed using an unpaired t-test (two tail) and Tukeys post test with significance being represented by P < 0.05. Statistical analyses were performed using InStat$^\text{TM}$ (GraphPad Software, San Diego, CA, USA). Selectivity ratios of enzymes were determined by the equation: vertebrate IC$_{50}$/ invertebrate IC$_{50}$.

### 3.3 Results

#### 3.3.1 Potency of AChE Inhibitors in Arthropods

The majority of compounds used for enzyme characterization act at the CS of AChE. Coumaphos oxon is a potent anticholinesterase against rBmAChE (Table 3-1), but is 43-fold less effective against rPpAChE (Table 3-1). Commercial carbamate insecticides (propoxur, bendiocarb, carbaryl, and bendiocarb) are highly potent inhibitors of both rBmAChE1 and rPpAChE (Table 3-1). For both rBmAChE and rPpAChE, the most active carbamate was carbofuran, whereas in the tick, propoxur was the least active, and for the sandfly it was carbaryl. Although commercial carbamates yielded fairly similar inhibition potencies for rPpAChE and rBmAChE, experimental carbamates possessed varying potencies against the two enzymes (Table 3-1). For rBmAChE1, experimental carbamates possess a range of approximately 25-fold, with 6 and 3 being the most and least potent inhibitor, respectfully. Compound 1 is the most potent experimental carbamate with a meta positioned side chain by approximately 5- to
10-fold, compared to 2 and 3, respectively. Compound 6 displayed the greatest potency for experimental inhibitors studied with an *ortho* positioned side chain. For *rPpAChE*, experimental inhibitors displayed a range of 18-fold with 1 and 7 being the most and least potent inhibitors, respectively. The *meta*-substituted compounds were of similar high potency, with compound 6 the only *ortho* carbamate of similar activity. This compound was also equipotent to both *rBmAChE1* and *rPpAChE* (Table 3-1). *rBmAChE1* was found to have low sensitivity (*IC*$_{50}$ = 220 µM) toward tacrine, a monovalent CS inhibitor with mid nanomolar blocking potency in all previous species tested, including *rPpAChE*. Eserine, a natural product CS cholinesterase inhibitor, was the most potent compound tested in this study, and possessed potencies toward *rBmAChE1* and *rPpAChE* differing by a factor of six (Table 3-1). The high potency of this carbamate may be attributable to its basic nitrogen functionality; protonation at assay pH confers a positive charge and greater attraction to the choline-binding site. Edrophonium, a reversible CS inhibitor, was considerably less potent than the carbamates (low micromolar range), and displayed similar mean *IC*$_{50}$ values in the low micromolar range against *rBmAChE1* and *rPpAChE*.

The two peripheral site inhibitors studied (tubocurarine and ethidium) displayed poor inhibition of *rBmAChE1*, but typical levels of inhibitory activity to *rPpAChE*. Tubocurarine inhibited no more than 15% of enzyme activity at 10$^{-3}$ M with *rBmAChE1*, and was over 25-fold more active against *rPpAChE* (Table 3-1). Ethidium was more active than tubocurarine in both species, and showed 5-fold greater potency for *rPpAChE* than *rBmAChE*.

Bivalent AChE inhibitors spanning both the CS and PS binding domains possessed reduced inhibition potency toward *rBmAChE1* and high potency toward *rPpAChE* (Table 3-1). BW284c51 was active at the low micromolar level on *rBmAChE1*, and was found to have 424-fold greater activity against *rPpAChE* (Table 3-1). E2020 (donepezil) displayed approximately three percent inhibition at 100 µM, and was therefore considered to be inactive on *rBmAChE1*. 
However, E2020 was a potent inhibitor of rPpAChE, as it was found to have an IC\textsubscript{50} value of ca. 100 nM (Table 3-1). To further understand the inhibitor profile of rBmAChE1 and rPpAChE, the enzymes were also studied using bis(n)-tacrine dimer series as structural probes to measure the distance between the CS and PS (Table 3-2). rPpAChE was found to be more sensitive to the entire tacrine dimer series when compared to rBmAChE1. Comparing IC\textsubscript{50} values across rBmAChE1 and rPpAChE, the differences in potency ranged from 297-fold for bis(8)-tacrine to 1493-fold for bis(10)-tacrine (Table 3-2). For rBmAChE1, the most potent tacrine dimer was found to be bis(8)-tacrine and the least potent was found to be bis(2)-tacrine. However, a different pattern of inhibition for rPpAChE produced less than a 2.5-fold difference between bis(7)-tacrine through bis(12)-tacrine, with the IC\textsubscript{50} values ranging from 2-5 nanomolar (Table 3-2).

3.3.2 Potency of AChE Inhibitors in Mammals

The two mammalian AChE enzymes studied, human and bovine, displayed similar inhibition potencies to CS-directed compounds, with the largest potency difference being ca. 10-fold to coumaphos oxon (Table 3-1). Commercial carbamate inhibitors also displayed little difference in potency values to the mammalian AChE enzymes with the largest difference being 1.9-fold (carbofuran). The most and least potent commercial CS inhibitors were found to be carbofuran and carbaryl, respectively, for both mammalian enzymes. Similarly, the experimental carbamates displayed little difference in IC\textsubscript{50} values. The largest difference was 3-fold (3) and potency ratios of most inhibitors neared unity. The most potent experimental carbamate for both enzymes was 1 (meta-substituted). A 2.7-fold difference was observed in potency with eserine, the second most potent CS inhibitor, with hAChE more sensitive than bovine AChE. Edrophonium was found to be a low micromolar inhibitor to both mammalian enzymes and hAChE was 1.6-fold more sensitive when compared to bovine (Table 3-1).
Mammalian enzymes were also found to possess similar sensitivities to both peripheral site inhibitors studied (Table 3-1). The potency differences between the two species was 1.05-fold and 1.4-fold for tubocurarine and ethidium, respectfully. Ethidium was found to be more potent than tubocurarine to both enzymes by approximately 2-fold.

Bivalent inhibitors, E2020 and BW284c51, were shown to be the most potent blockers of both mammalian enzymes, with IC_{50} values in the low nanomolar range (Table 3-1). E2020 was the most active inhibitor to both mammalian enzymes, with nearly equipotent IC_{50} values against hAChE and bovine AChE. Although 2- to 4-fold less potent than E2020, BW284c51 was still the second most potent bivalent inhibitor to the mammalian enzymes, with inhibition values differing by 2.1-fold across mammal species.

### 3.3.3 Inhibitor Selectivity Across Mammals and Arthropods

SR values are used to express in vitro selectivity differences between mammalian and arthropod enzymes, as shown in Table 3-1. For rBmAChE1, the most selective standard carbamate was carbaryl for both human and bovine enzymes. Otherwise, commercial carbamates were shown to have a large range of enzyme selectivity, varying from 8- to 174-fold for hAChE and 12- to 163-fold for bovine AChE. Edrophonium was found to be negatively selective for both the human and bovine AChE enzymes, as they were more active on the mammalian enzymes when compared to rBmAChE1. Eserine was found to be highly selective for rBmAChE1 over bovine AChE (123-fold) and moderately selective over hAChE (46-fold). The SR of experimental carbamates for rBmAChE1 were shown to be highly variable and ranged from 1-fold to 360-fold for hAChE and 4-fold to 338-fold for bovine AChE (Table 3-1). The most potent experimental inhibitor, 6, presented the largest SR (338-fold) for bovine AChE and was also found to be very selective against hAChE with an SR of 342. The lowest SR observed of experimental carbamates for rBmAChE1 and bovine and hAChE was 3, with SR values of 4-
fold and 1-fold, respectively. Coumaphos oxon was found to have SR of 104-fold and 11-fold for bovine and hAChE, respectively. The SR values for 6 are a 3.3-fold (bovine AChE) and 31-fold (hAChE) increase over that of the currently used acaricide, coumaphos oxon. PS and bivalent inhibitors were both found to be negatively selective, as they inhibited the mammalian enzyme with greater efficacy compared to rBmAChE1.

The hAChE selectivity (Table 3-1) of the experimental carbamates with PpAChE was found to range from 23-fold (1) to 611-fold (7). Propoxur was found to be most selective commercial carbamate with an SR of 21-fold, whereas 6 was found to have an SR of 366-fold, a 17-fold increase over propoxur. Edrophonium was found to be negatively selective for PpAChE, whereas eserine was 7.6-fold selective, 3-fold less than the least selective experimental carbamate. PS inhibitors were both found to be poorly selective with SR values near unity. A ten-fold difference in SR was observed between the bivalent inhibitors with E2020 being negatively selective for PpAChE and BW284c51 being non-selective (SR = 1.1).

3.3.4 Homology Modeling and Site Directed Mutagenesis (W384F) of rBmAChE1

Homology models were constructed by Dr. Max Totrov. Homology model of the BmAChE1 was constructed, and the percentage identity of the template and target sequence was 42%. The alignment contained 8 insertions/deletions. All but one of the indels were remote from the CS/PS, with a loop three residues shorter being on the outer rim of the PS (N336-V340). Overall backbone RMSD to the template was 1.35A. The catalytic and peripheral sites as well as the gorge were inspected and compared to X-ray structures of the complexes of tacrine (PDB ID 1ACJ) and E2020 (PDB ID 1EVE). The model revealed the organization of the CS, gorge and PS that was overall similar to other species, but several distinctive features were observed. Firstly, AChE from other species typically has a phenylalanine or tyrosine residue in the position corresponding to W384. Review of the inhibitor complex structures revealed that
the phenylalanine side chain is able to adopt alternative orientations, either enlarging the catalytic site so that tricyclic ligands such as tacrine can be accommodated, or expanding the gorge when bulkier moieties are present there, as is the case with E2020. On the other hand, larger W384 side-chain in BmAChE1 fills most of the space occupied by either of the phenylalanine conformers. Another significant difference observed is that generally highly conserved tryptophan residue in the PS (W286 in human enzyme) is substituted by T335.

Inhibition potencies of AChE inhibitors to the rBmAChE1 (W384F) mutant enzyme and a comparison to rBmAChE1 are shown in Table 3-3. The mutated rBmAChE1 (W384F) enzyme displayed a statistically significant increase in inhibition when compared to rBmAChE1 wildtype with the inhibitors tacrine, BW284c51, and E2020. Tacrine and BW284c51 was found to be 6.4-fold and 8-fold more potent to the mutated enzyme (W384F) when compared to rBmAChE1 wildtype. E2020 displayed a > 40-fold increase in potency to the mutated enzyme, but an exact value was not able to be determined due only 3% of the rBmAChE1 wildtype enzyme being inhibited at 100 μM. Propoxur was the only standard or experimental AChE inhibitor to show a statistically significant increase (1.4-fold) in IC\textsubscript{50} value between the wildtype and mutant enzymes. The near unity ratios of commercial and experimental inhibitors indicate the mutation had no effect on the catalytic activity of the mutated enzyme when compared to the wildtype.

3.4 Discussion

*Rhipicephalus (Boophilus) microplus* and *Phlebotomus papatasi* are both of great concern due to their ability to vector diseases. *Pp* present significant issues in numerous countries as it is the primary vector for zoonotic cutaneous leishmaniasis transmission to humans and has been the major cause of disease morbidity among United States military personnel (Desjeux, 2001; Pehoushek et al., 2004; Willard et al., 2005). *Bm* populations are of veterinary concern as they are a potentially deadly pest of cattle and induce large economic burdens on
cattle farmers in southwestern United States and Mexico (Jonsson et al., 1998). Chemical insecticides have been the primary mechanism for control of both disease vectors throughout recent history. However, recent reports of insecticide resistance have amplified the need for the design of novel chemicals to control these vector populations (Rosario-Cruz, 2005; Morgan et al, 2009; Hassan et al., 2012). To further the design of novel chemicals, an understanding of the target site protein is vital to determine protein structure and to the development of inhibitor specificity.

CS inhibitors were highly potent, with IC$_{50}$ values extending into the low nanomolar range, indicating the W384/Y337 substitution has little bearing on the activity of smaller, monovalent molecules. The experimental methylcarbamates possessed side chains in the meta or ortho positions. Compound 1 was found to be the most potent inhibitor with a meta substituted side chain. Inhibition potencies decreased 5-fold and 10-fold for 2 and 3, respectively, when compared to 1. This suggests the rBmAChE1 enzyme is not capable of accommodating the larger trimethylsilyl group, indicating the meta substituted side chains must be smaller in size to effectively inhibit the enzyme. Ortho-substituted phenyl methylcarbamates 4-7 were found to be highly potent to the rBmAChE1 enzyme, and were most potent with thiol substituted side chains. Compound 7 possesses a 2-ethylbutoxy substituted side chain, and was less potent than all three thiolalkyl-substituted side chains, indicating that its lipophilicity or polarizability may alter interactions of the inhibitor at the rBmAChE1 acyl site.

A different inhibition pattern was observed with PpAChE as it is more sensitive to meta-substituted phenyl methylcarbamates 1-3 versus meta-substituted phenylmethyl carbamates 4, 5, and 7. One exception was found to be 6, as it was the second most potent experimental carbamate, and was 6-fold more active than any other ortho-substituted carbamate studied. The high potency of 6 to rBmAChE1 and rPpAChE is exceptional, as it is 7-fold more potent when
compared to AgAChE (IC$_{50}$: 104 nM), the enzyme species it was designed to target (Jiang et al., submitted).

The activated form of coumaphos, an anticholinesterase insecticide currently used in Bm control programs, possesses 100-fold selectivity for BmAChE1 over bovine AChE and a mere 10-fold selectivity over hAChE. Although these selectivity values are higher than other anticholinesterases, 6 was shown to be equipotent (14 nM) to coumaphos oxon and possess 338-fold and 342-fold selectivity over bovine AChE and hAChE, respectively. Selectivity of experimental carbamates that possess ortho substituents was also excellent for rBmAChE1 compared to the mammalian AChE enzymes. This selectivity was abolished to less than ten-fold with meta-substituted side chains due to a lower potency to rBmAChE1, suggesting the design of future N-methylcarbamates for Bm control should utilize ortho-substitutions. Compound 6 was also found to be highly selective for PpAChE over hAChE with a SR of 366, while standard carbamates were found to have SR of less than 20-fold for PpAChE over hAChE. This finding indicates that the experimental carbamates are a viable control method for Pp populations in locations of close proximity to humans, such as insecticide treated nets or indoor residual spraying.

rBmAChE1 was found to be over 1000-fold less sensitive to tacrine when compared to all other species studied, indicating a unique catalytic site in BmAChE. Molecular homology models indicate that there are two significant paralogous substitutions that inhibit ligand binding in BmAChE1, W384/Y337 and T335/W286 (Bm/human numbering) (Figure 3-2). Typically, phenylalanine or tyrosine is analogous to the W384 residue in BmAChE1. In Tc, F330 is analogous to W384 in the tick and is thought to possess two orientations to accommodate various ligands. These two orientations are shown in Figure 3-2. However, the W384 residue in BmAChE1 possesses a larger side chain than Y337 in hAChE (F330 in TcAChE). The model
suggests that steric clashes of the tryptophan side chain with bulky ligands in the CS (tacrine) or gorge (E2020) would be resolved by the re-orientation of the tyrosine side chain in AChE of other species, but cannot be completely avoided with the W384 of BmAChE1. This structural clash contributes to the significantly reduced affinity of these inhibitors, although the difference in potency for tacrine can be only partially accounted for by mutation W384F (Table 3-3).

Presumably, the T335/W286 substitution also had a large contribution to the reduced potency of the tacrine dimer series. The monomeric tacrine was among the least active inhibitor in the series, with bis(8)-tacrine being the most potent at an IC<sub>50</sub> value of about 1 uM. The pattern of inhibition for rBmAChE was similar to hAChE (Anderson et al., 2009), as the IC<sub>50</sub> decreased with increasing tether length to bis(8)-tacrine, and then increased again as tether length neared 12 methylenes. The chicken enzyme is known to be missing Y70, Y121, and W270, three principal PS residues, and is therefore considered to be devoid of a PS (Eichler et al., 1994). Interestingly, rBmAChE1 was found to be 16-fold (A9A) to 341-fold (A4A) less sensitive to the tacrine dimer series when compared to chicken AChE (Mutunga, 2011), suggesting the T335/W286 substitution (Bm/human numbering) potentially provides a blockade effect or steric hindrance that serves to prevent adequate binding of bivalent ligands to the CS and PS sites. However, it is unlikely that the peripheral site is absent in rBmAChE1, as ethidium bromide inhibited the enzyme with nearly the same potency values seen in numerous other species. Additionally, the pattern of inhibition (increased potency with increasing tether length to bis(8)-tacrine, then decreasing potency) indicates the presence of dual binding.

The rPpAChE enzyme yielded a different response when compared to rBmAChE1, AgAChE and hAChE. The monomeric tacrine was found to be among the least potent of the tacrine dimer series, but was similar in potency to every species studied (IC<sub>50</sub> ca. 200 nM), excluding BmAChE. However, the pattern of inhibition was different than rBmAChE1 as the
IC$_{50}$ decreased with increasing tether length, but did not increase again as the tether link neared 12 methylenes. *Drosophila melanogaster* AChE, an *ace-2* encoding insect, has also been shown to have this pattern of inhibition to the tacrine dimer series (Mutunga, 2011). Insects utilizing the *ace-2* gene to encode the functional AChE unit may likely possess a different protein structure than *ace-1* insects that is capable of accommodating larger tether lengths over shorter tethers.

In an effort to validate the homology models, site directed mutagenesis was performed to study the effect the W384/Y337 substitution has on the inhibitor potency to the *rBm*AChE1 enzyme. The mutation performed was a W384F mutation due to phenylalanine possessing a smaller structural size when compared to tryptophan, allowing a closer resemblance to tyrosine, which is found in the majority of other enzymes, including *hAChE*. Statistically significant increases in potency were observed with tacrine and the two bivalent inhibitors, BW284c51 and E2020. Although the increase in potency to the mutated enzyme with tacrine and bivalent inhibitors validates the model of W384 preventing access of larger ligands to the acyl site, it does not fully account for the decreased *rBm*AChE1 sensitivity, as the W384F mutation did not decrease the IC$_{50}$ values to that observed in other species. One would expect an additional 172-fold increase in potency for tacrine if the mutation accounted for all of the decreased inhibitor potency. Therefore, it is plausible to suggest other paralogous substitutions are present in conjunction with W384/Y337 (*Bm/human* numbering), allowing for a unique *Bm*AChE1 gorge geometry and therefore, a constricted entry to the acyl site. For instance, molecular homology models suggest the T335/W286 (*Bm/human* numbering) substitution in the *Bm* peripheral site could account for a reduced sensitivity to peripheral site and bivalent inhibitors. The T335/W286 substitution in *rBm*AChE1 could potentially disrupt the transient binding mechanism
of the PS (Szegletes et al., 1999) and effectively reduce the potency of acyl site inhibitors through allosteric effects at the CS.

To conclude, the findings of the current study have significant implications for the future design of selective and resistance-mitigating inhibitors for the control of vectored diseases. The non-selective inhibitor, tacrine, yielded a 1000-fold difference in the inhibition profile for \(Bm\)AChE1 over \(Pp\)AChE. This indicates a unique AChE gorge geometry drastically different than that of \(Pp\)AChE and all other enzyme species studied. Interestingly, despite the structural differences of \(Bm\)AChE1, the highly selective experimental carbamate 6 is nearly equipotent toward \(Bm\)AChE1 and \(Pp\)AChE and is more potent toward these two enzyme species than \(Ag\)AChE, the species it was designed to target. The high potency toward \(Bm\)AChE1 and \(Pp\)AChE in conjunction with the low mammalian activity provides an attractive alternative and superior insecticide for \(Bm\) and \(Pp\) control.
Figure 3-1. Structures of experimental methylcarbamate inhibitors used in this study.
<table>
<thead>
<tr>
<th></th>
<th>tBmAChE IC₅₀ (nM (95% CI))</th>
<th>tPpAChE IC₅₀ (nM (95% CI))</th>
<th>hAChE IC₅₀ (nM (95% CI))</th>
<th>Bovine AChE IC₅₀ (nM (95% CI))</th>
<th>SR¹</th>
<th>SR²</th>
<th>SR³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumaphos oxon</td>
<td>10 (2-17)</td>
<td>430 (349-511)</td>
<td>111 (103-121)</td>
<td>1,038 (875-1201)</td>
<td>104</td>
<td>11</td>
<td>0.26</td>
</tr>
<tr>
<td>Propoxur</td>
<td>33 (20-46)</td>
<td>89 (50-126)</td>
<td>1,442 (2430-3131)</td>
<td>1,835 (1289-2381)</td>
<td>55</td>
<td>43</td>
<td>21</td>
</tr>
<tr>
<td>Carbofuran</td>
<td>5 (3-8)</td>
<td>8 (2-14)</td>
<td>38 (30-45)</td>
<td>73 (48-98)</td>
<td>15</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>16 (5-27)</td>
<td>167 (126-208)</td>
<td>2,780 (2430-3131)</td>
<td>2,605 (2343-2868)</td>
<td>163</td>
<td>174</td>
<td>17</td>
</tr>
<tr>
<td>Bendio carb</td>
<td>16 (7-24)</td>
<td>15 (14-16)</td>
<td>182 (113-250)</td>
<td>195 (173-216)</td>
<td>12</td>
<td>11</td>
<td>12</td>
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<tr>
<td>Eserine</td>
<td>1.5 (0.5-2.5)</td>
<td>9 (4-14)</td>
<td>69 (54-84)</td>
<td>185 (145-224)</td>
<td>123</td>
<td>46</td>
<td>7.6</td>
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<tr>
<td>Edrophonium</td>
<td>2,425 (1941-2910)</td>
<td>1,178 (584-1771)</td>
<td>1,081 (7774-1387)</td>
<td>1,799 (1506-2091)</td>
<td>0.74</td>
<td>0.45</td>
<td>0.92</td>
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<td>BW284C51</td>
<td>12,723 (12,423-13,024)</td>
<td>30 (20-39)</td>
<td>33 (18-46)</td>
<td>16 (11-21)</td>
<td>0.001</td>
<td>0.003</td>
<td>1.1</td>
</tr>
<tr>
<td>Tacrine</td>
<td>220,766 (171,378-270,155)</td>
<td>205 (168-240)</td>
<td>213 (122-304)</td>
<td>187 (143-229)</td>
<td>0.001</td>
<td>0.001</td>
<td>1.04</td>
</tr>
<tr>
<td>E2020</td>
<td>3 % inhibition at 10⁻⁴M</td>
<td>92 (69-114)</td>
<td>7 (3-10)</td>
<td>&lt;0.01</td>
<td></td>
<td>&lt;0.01</td>
<td>0.08</td>
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<tr>
<td>Tubocurarine</td>
<td>15 % inhibition at 10⁻³M</td>
<td>38,890 (31034-46746)</td>
<td>57,606 (48625-66588)</td>
<td>54,396 (49236-59558)</td>
<td>&lt;0.05</td>
<td>&lt;0.06</td>
<td>1.5</td>
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<td>Ethidium Bromide</td>
<td>77,710 (68,349-87,071)</td>
<td>14,136 (9391-18882)</td>
<td>22,886 (14600-31174)</td>
<td>323,43 (26316-38371)</td>
<td>0.42</td>
<td>0.3</td>
<td>1.6</td>
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<tr>
<td>1</td>
<td>37 (18-62)</td>
<td>10 (4-16)</td>
<td>233 (154-311)</td>
<td>259 (242-277)</td>
<td>7</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>190 (150-230)</td>
<td>22 (13-30)</td>
<td>539 (484-593)</td>
<td>1,053 (896-12210)</td>
<td>5</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>364 (256-337)</td>
<td>16 (8-24)</td>
<td>451 (431-470)</td>
<td>1,357 (1284-1430)</td>
<td>4</td>
<td>1</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>25 (10-39)</td>
<td>150 (90-209)</td>
<td>8,035 (7743-8327)</td>
<td>6,366 (6027-6704)</td>
<td>255</td>
<td>321</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>77 (56-96)</td>
<td>100 (73-126)</td>
<td>10,906 (10451-11362)</td>
<td>8,955 (6055-12810)</td>
<td>116</td>
<td>142</td>
<td>109</td>
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<tr>
<td>6</td>
<td>15 (3-26)</td>
<td>14 (11-15)</td>
<td>5,127 (4871-5383)</td>
<td>5,073 (4772-5373)</td>
<td>338</td>
<td>342</td>
<td>366</td>
</tr>
<tr>
<td>7</td>
<td>312 (256-366)</td>
<td>184 (137-229)</td>
<td>112,600 (72,489-152,711)</td>
<td>92,016 (85,035-98,998)</td>
<td>295</td>
<td>360</td>
<td>611</td>
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</table>
Table 3-2. Tacrine and tacrine dimer inhibition of tick and sandfly AChE.

<table>
<thead>
<tr>
<th>Compound</th>
<th>rBmAChE1 IC₅₀ (nM; 95% CI)</th>
<th>PpAChE IC₅₀ (nM; 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tacrine</td>
<td>220,766 (171,378-270,155)</td>
<td>205 (168-240)</td>
</tr>
<tr>
<td>bis(2)-tacrine</td>
<td>190,095 (162,000-217,828)</td>
<td>151 (132-166)</td>
</tr>
<tr>
<td>bis(3)-tacrine</td>
<td>131,917 (119840-156,091)</td>
<td>139 (100-161)</td>
</tr>
<tr>
<td>bis(4)-tacrine</td>
<td>45,740 (33,582-56,442)</td>
<td>103 (87-128)</td>
</tr>
<tr>
<td>bis(5)-tacrine</td>
<td>11,748 (9,061-15,910)</td>
<td>25 (15-36)</td>
</tr>
<tr>
<td>bis(6)-tacrine</td>
<td>9,421 (6209-13017)</td>
<td>9 (5-15)</td>
</tr>
<tr>
<td>bis(7)-tacrine</td>
<td>2919 (2198-3675)</td>
<td>5 (2-8)</td>
</tr>
<tr>
<td>bis(8)-tacrine</td>
<td>892 (510-1109)</td>
<td>3 (0.5-6)</td>
</tr>
<tr>
<td>bis(9)-tacrine</td>
<td>2046 (1495-2583)</td>
<td>3 (0.5-5)</td>
</tr>
<tr>
<td>bis(10)-tacrine</td>
<td>2986 (2687-3384)</td>
<td>2 (0.3-5)</td>
</tr>
<tr>
<td>bis(12)-tacrine</td>
<td>4126 (3368-4892)</td>
<td>3 (0.6-6)</td>
</tr>
</tbody>
</table>
Table 3-3. IC\textsubscript{50} values of AChE inhibitors with the mutated r\textit{Bm}AChE1 (W384F) enzyme compared to r\textit{Bm}AChE1 wildtype. IC\textsubscript{50} values are expressed in nanomolar units and are shown as means (n = 3). Asterisks represent statistical significance at P < 0.05 when compared to the wild type enzyme values (Table 3-1).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>r\textit{Bm} W384F AChE1 - IC\textsubscript{50} (nM; 95% CI)</th>
<th>r\textit{Bm}AChE1 IC\textsubscript{50} / W384F IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumaphos oxon</td>
<td>9 (3-15)</td>
<td>1.1</td>
</tr>
<tr>
<td>Propoxur</td>
<td>47 (36-57)*</td>
<td>0.7</td>
</tr>
<tr>
<td>Carbofuran</td>
<td>6 (3-10)</td>
<td>0.8</td>
</tr>
<tr>
<td>Bendiocarb</td>
<td>18 (10-25)</td>
<td>0.9</td>
</tr>
<tr>
<td>BW284C51</td>
<td>1570 (1481-1658)*</td>
<td>8.1</td>
</tr>
<tr>
<td>Tacrine</td>
<td>34,470 (28175-40765)*</td>
<td>6.4</td>
</tr>
<tr>
<td>E2020</td>
<td>2298 (1385-3211)*</td>
<td>43.5</td>
</tr>
<tr>
<td>1</td>
<td>53 (36-69)</td>
<td>0.7</td>
</tr>
<tr>
<td>6</td>
<td>23 (15-31)</td>
<td>0.7</td>
</tr>
<tr>
<td>7</td>
<td>256 (244-266)</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Figure 3-2. Superposition views of tacrine (A) and E2020 (B) complexes onto the model of BmAChE1. F330 is shown to exist in two orientations: 1) conformation adopted in complex with tacrine is shown in magenta and 2) the conformation adopted in complex with E2020 is shown in green. Conserved W84 is shown at the bottom of the active site for orientation.
CHAPTER 4
THE TOXICITY AND MODE OF ACTION OF N,N-DIETHYL-META-TOLUAMIDE (DEET) ON THE NERVOUS SYSTEM

Abstract: Recent studies have raised the possibility that N,N-diethyl-3-methylbenzamide (DEET) is an acetylcholinesterase (AChE) inhibitor and that this action may contribute to its effects in insects, and cause risk of toxicity in exposed humans. DEET causes disrupted posture and hyperexcitation that is distinct from the anticholinesterase propoxur, as well as lethality in mosquitoes at topical doses in the microgram range (2-4 μg), but DEET is an extremely poor AChE inhibitor in mosquitoes (<10% inhibition), even at a concentration of 10 mM.

Neurophysiological recordings were performed to determine the effect of DEET on the insect nervous system, and compared to toluene and lidocaine, structurally related compounds with anesthetic actions. DEET was found to have neuroexcitatory effects on the CNS in the micromolar range (EC\textsubscript{50}: 120 μM), over 1000-fold less potent than propoxur. Phentolamine was found to completely block the CNS neuroexcitation of DEET and octopamine, but was not found to be an effective blocker of propoxur.

DEET and lidocaine blocked neuromuscular transmission in housefly larvae, as well as Na\textsuperscript{+} and K\textsuperscript{+} channels in rat cortical neurons. Taken together, these findings suggest DEET is likely targeting octopaminergic synapses and not acetylcholinesterase to induce neuroexcitation and presumably toxicity. The ion channel blocking action of DEET would be consistent with the numbness experienced after inadvertent application to the lips or mouth.

4.1 Introduction

The insect repellent DEET (N,N-diethyl-3-methylbenzamide) is used more often than any other mosquito repellent, with greater than 200 million people users worldwide (Moore and Debboun, 2007). There is still much debate as to the mode of repellent action and molecular
targets of DEET. One argument suggests DEET blocks olfactory sensory neurons that detect attractants, such as carbon dioxide and 1-octen-3-ol (Davis and Sokolove, 1976; Ditzen et al., 2008; Dogan et al., 1999). Other research has found that insects detect DEET through olfactory mechanisms that elicit avoidance behavior (Carroll et al., 2005; Syed and Leal, 2008). There is experimental evidence for both mechanisms, potentially indicating that DEET does not affect one specific molecular target, but can provide repellency through multiple modes of action. An understanding of the molecular mode of action for both repellency and toxicity is essential for the development of novel repellents and for continued control of vector borne diseases.

Although the molecular mode of action of repellency by DEET remains elusive, there has been interest in the toxicological profile of DEET since it has been shown to have insecticidal properties (Moss, 1996; Licciardi et al., 2006). Moss (1996) also speculated, based on comparisons of synergism effects of DEET, amitraz and chlordimeform, that DEET may have some toxic actions similar to those of formamidine pesticides. Large oral doses of DEET (blood concentration of 1 mmol/litre) have been shown to lead to nausea, vomiting, bradychardia, and seizures in humans (Ellenhorn, 1997). Documentation also exists of DEET being potentially toxic to humans when combined with pesticides (Lipscomb et al., 1992; Clem et al., 1993), and recent literature suggests the toxic action is due to anticholinesterase properties (Corbel et al., 2009).

The majority of human exposure to DEET is through contact exposure, therefore limiting the applicability of toxicity data via oral exposure. Contact exposure of DEET also has the potential for negative effects to humans, as exposure can lead to numbness and redness of the affected area (Reuveni and Yagupsky, 1982). The numbing sensation is similar to those observed with local anesthetics, such as lidocaine, raising the question if DEET is acting on
specific ion channels to yield an anesthetic-like effect versus possessing anticholinesterase properties.

The objective of this study was to determine the mode of action of DEET neurotoxicity and compare its action to known anticholinesterases, local anesthetics, and octopaminergic chemicals with the goal of providing insights into the specific targets of DEET with respect to the acute toxicity.

4.2 Materials and Methods

4.2.1 Inhibitors, Solvents, and Assay Reagents

The inhibitors DEET (N,N-diethyl-3-methylbenzamide), lidocaine, toluene, cesium, and propoxur were all purchased from Sigma-Aldrich (St. Louis, MO, USA) and were ≥ 98% pure. Structures of these inhibitors are shown in Figure 4-1. The solvent, dimethyl sulfoxide (DMSO) was also purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular sieve OP type 3Å was purchased from Sigma (St. Louis, MO, USA) and were used to prevent water absorption within the DMSO stock. Fifty beads were added into a 100 mL stock solution. These sieves have a diameter of ~2 mm, a pore size of 3Å, and a water absorbing capacity of ≥ 15%.

Ellman assay (Ellman et al., 1961) reagents are composed of acetylthiocholine iodide (ATCh)(≥ 99% purity), 5,5'-dithiobis-(2-nitro)benzoic acid (DTNB)(99% purity), and sodium phosphate buffer, all of which were purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular sieve OP type 3Å was purchased from Sigma (St. Louis, MO, USA) and were used to prevent water absorption within the DMSO stock. Fifty beads were added into a 100 mL stock solution. These sieves have a diameter of ~2 mm, a pore size of 3Å, and a water absorbing capacity of ≥ 15%.

4.2.2 Enzyme Sources, Insects, and Neuronal Cells

In vitro biochemical assays utilized five acetylcholinesterase enzymes: An. gambiae homogenate (AgAChE; wild type G3 strain, cultured in the Emerging Pathogens Institute, University of
Florida, Gainesville, FL, USA from eggs provided by the Center for Disease Control, Atlanta, GA, USA), *Ae. aegypti* (AaAChE; cultured in the CMAVE, USDA-ARS, Gainesville, FL, USA), *Musca domestica* (MdAChE; cultured in Department of Entomology and Nematology, Medical Entomology Laboratory, University of Florida, Gainesville, FL, USA), and *Drosophila melanogaster* (DmAChE; Oregon-R strain, cultured in the Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA) and recombinant human (*h*AChE; lyophilized powder, Sigma C1682, St. Louis, MO, USA). Homogenate enzymes were prepared from groups of ten non-blood fed adult female mosquitoes (or five whole bodied *Drosophila*, three *Musca* heads) homogenized in 1 mL of ice-cold sodium phosphate buffer (0.1 M sodium phosphate, pH 7.8,) with an electric motor driven glass tissue homogenizer. The homogenate was centrifuged at 5000 x g using a Sorvall Fresco refrigerated centrifuge at 4° C for 5 minutes. The supernatant was used as the enzyme source for the assay, and all enzyme preparations contained 0.3 % (v/v) triton X-100 and 1 mg/ml BSA. Prior to use in assay, the recombinant human enzyme was diluted 500-fold into sodium phosphate buffer containing triton X-100 and 1 mg/ml BSA.

Insects used for in vivo toxicity assays were obtained from sources listed above. In addition, *An. gambiae* (AKRON strain) was cultured at the University of Florida, Emerging Pathogens Institute, Gainesville, FL, USA from eggs supplied from stocks maintained at the Center for Disease Control (Atlanta, GA, USA). Rat neuronal cortex cells were purchased from Invitrogen (Grand Island, NY, USA), plated on 35 mm glass cover slips, and maintained in primary neuron basal medium (PNBM) without L-glutamine at 38°C until used for patch clamp studies.
4.2.3 Enzyme Inhibition Assays

Inhibition of AChE was determined using the Ellman assay (Ellman et al., 1961) and was based on the method outlined in Carlier et al., 2008. Enzyme solution (10 µL) was added to each well of the 96-well micro assay plate along with 20 µL of inhibitor, dissolved in DMSO, and 150 µL of sodium phosphate buffer. The plate was incubated at 25°C for ten minutes. Ellman assay reagents, ATCh (0.4 mM, final concentration) and DTNB (0.3 mM, final concentration), were prepared fresh and 20 µL was added to the enzyme to initiate the reaction. Changes in absorbance were recorded by a DYNEX Triad spectrophotometer (DYNEX Technologies, Chantilly, VA, USA) at 405 nm. Enzyme concentrations used were within the linear range, therefore eliminating the need for protein quantification. IC\textsubscript{50} values for each species were calculated by nonlinear regression from eight inhibitor concentrations using GraphPad Prism\textsuperscript{TM} (GraphPad Software, San Diego, CA, USA). The nonlinear regression equation used was as follows:

\[ Y = \text{bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\log\text{EC}_{50} - x)\times\text{Hillslope})}); \]

\[ \text{where } x = \text{the logarithm of the concentration and } Y = \text{the response.} \]

\[ Y \text{ starts at the top (normalized 100\%) and approaches the bottom (0\%) with a sigmoid shape.} \]

4.2.4 Toxicity Assays

Topical toxicity bioassays were performed based on the method of Pridgeon et al (2008). Briefly, insects were chilled on ice for 3 min, during which the appropriate volume (200 nL for mosquitoes, 1 µL for Musca) of chemical (dissolved in 95% Ethanol) was applied onto the abdomen of insect using a handheld Hamilton® microapplicator. For each inhibitor, five doses were applied on ten insects each and repeated three times. An ethanol-only treatment was
included in each experiment as a negative control. Insects were transferred into paper cups covered with netting and supplied with free access to sugar water for the duration of the experiment. Mortality was recorded at the 24-hour time point. Mortality data was pooled and analyzed by log-probit using Poloplus® to determine 24 hr LD<sub>50</sub> values. Three LD<sub>50</sub> values were obtained and the mean LD<sub>50</sub> value was calculated and used for statistical analysis.

Diet toxicity assays were performed on <i>Drosophila melanogaster</i>. Flies were fasted for 8 hours prior to initializing the experiment. Flies were transferred into a container that contained a cotton ball infused with a sugar/inhibitor solution. For each inhibitor, one dose (1 mg/ml) was applied on twenty-five flies and was replicated five times. Mortality was recorded at the 24-hour time point.

Contact toxicity bioassays were performed on both strains of <i>An. gambiae</i>, G3 (susceptible) and AKRON (pyrethroid and anticholinesterase resistance) with the use of the WHO protocol (WHO, 1981). Adult female mosquitoes were 2-5 days of age and were non-blood fed at the time of experimentation. Five concentrations of inhibitor dissolved in ethanol were prepared and treated by applying 2 mL of each concentration on a 180 cm<sup>2</sup> (12 cm x 15 cm) paper. Papers were left to dry for 24 hours prior to use. Mosquitoes were chilled for three minutes on ice, after which 25 females were placed in the WHO kit holding chamber to recover for one hour. Mosquitoes were then moved to the treatment chamber, which contain the treated paper, and exposed for one hour. After the one-hour exposure, the mosquitoes were transferred back to the holding chamber and maintained on 10% sugar solution for 24 hrs. Each concentration was repeated in triplicate. Mortality was recorded 24 hours post treatment and an LD<sub>50</sub> was calculated using Poloplus® (LeOra Sofware Company, Petaluma, CA, USA). Three LD<sub>50</sub> values were obtained and the mean LD<sub>50</sub> value was used for statistical analysis.
For all toxicity assays, control mortality was corrected for using Abbots formula (Abbot, 1925): Corrected percent mortality = (\% alive in control - \% alive in treated) / \% alive in control

4.2.5 Electrophysiological Studies

4.2.5.1 Saline

Suction electrode recordings were performed on the central and peripheral nervous systems of larval *Musca domestica*. The central nervous system recordings used saline containing: 157 mM NaCl, 3 mM KCl, 2 mM CaCl₂, and 4 mM HEPES. The peripheral nervous system and neuromuscular junction recordings were performed with saline containing: 140 mM NaCl, 0.75 mM CaCl₂, 5 mM KCl, 4 mM MgCl₂, 5 mM NaHCO₃, and 5 mM HEPES. Both saline solutions were held at a pH of 7.25.

Patch clamp experiments were performed on rat neuronal cortex cells and used a variety of different saline solutions based upon the ion channel of interest. Extracellular solution was the same regardless of the target ion channel and contained: 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM HEPES (pH = 7.4). Intracellular patch solution for potassium channel recordings consisted of: 140 mM KF, 10 mM NaCl, 2 mM MgCl₂, and 10 mM HEPES (pH = 7.2). Intracellular patch solution for sodium recordings consisted of: 140 mM CsCH₃SO₃, 10 mM NaCl, 2 mM MgCl₂, 10 mM HEPES (pH = 7.2).

4.2.5.2 Musca CNS recordings

Neurophysiological recordings were performed on third instar *Musca domestica* larvae, and were based on methods described in Bloomquist et al. (1991). Electrodes/pipettes were pulled from borosilicate glass capillaries with filament (outer diameter 1.0 mm, inner diameter 0.8 mm (Sutter Instrument, Novato CA, USA) on a P-1000 Flaming/Brown micropipette puller
(Sutter Instrument). The settings on the pipette puller are as follows: Heat = 565, pull = 0, velocity = 35, time = 200, pressure = 500, RAMP = 561.

The CNS was first excised from the larvae and placed in a separate dish containing the appropriate physiological saline (200 uL), described in section 4.2.5.1. A drawing of the CNS dissection and recording arrangement is shown in Figure 4-2a. The CNS was manually transected posterior to the cerebral lobes to disrupt the blood-brain barrier and enhance chemical penetration into the CNS (Bloomquist et al., 1991). After dissection was complete, peripheral nerve trunks were pulled into a recording suction electrode. This allowed amplification and digitization of the electrical activity originating from the CNS. The signal was fed into an amplifier that quantified individual spikes and converted them into a rate, expressed in Hz (MacLab, ADInstruments, Colorado Springs, CO, USA). Activity was monitored using LabChart 7 for a ten minute time period to establish a constant baseline firing rate, as the spike frequency typically increased from 0 to 10 minutes before stabilization.

After a constant baseline was established, the CNS preparation was directly exposed to test compound by adding 200 uL of the chemical to the bath containing 200 uL of saline. The final concentration of solvent in the bath was 0.1% DMSO. Each concentration was recorded for three to five minutes prior to the addition of the next inhibitor concentration.

Mean spike frequencies for each concentration were used to construct dose response curves. Dose-response curves were used to determine the EC$_{50}$ values and were calculated by nonlinear regression (variable slope) using GraphPad Prism™ (GraphPad Software, San Diego, CA, USA) in a manner similar to that for AChE inhibition. Each inhibitor concentration was replicated 3-5 times.
4.2.5.3 Musca sensory recordings

As with the CNS recordings, the sensory recordings were also performed on third instar Musca domestica larvae with the same suction electrodes. The fat body, digestive system, and central nervous system were removed from the body to ensure non-synaptic activity was recorded. After dissection was complete, a peripheral nerve trunk containing sensory nerve axons was drawn into a recording suction electrode. A drawing of the sensory recording system is shown in Figure 4-2b. Activity was monitored for a 5 minute time period to establish a constant baseline of spike activity. The inhibitors were applied directly to the larval body cavity and the final concentration of solvent (usually DMSO) never exceeded 0.1%. Each concentration was recorded for three minutes or until the spike frequency became constant.

Mean spike frequencies for each concentration were analyzed identically to those of the CNS recordings.

4.2.5.4 Neuromuscular junction recordings

Third instar larvae were prepared in the manner as described in the Musca sensory recordings (section 4.2.5.3). In short, the maggot was immobilized with pins, and the nervous and musculature systems were exposed. Saline was identical to that used in the sensory recordings and is described in section 4.2.5.1. The lateral nerves were severed from the base of the CNS. A lateral nerve trunk was drawn into a suction electrode (which was filled with saline). Stimuli were applied at 1 volt with a repetitive frequency of 0.2 milliseconds to elicit a contraction from the longitudinal muscle. The stimulated muscle was then impaled with a recording glass capillary microelectrode filled with 1 M KCl to record the effect on the evoked EPSP and membrane potential. The resting membrane potential of all muscles prior to recording ranged between -30 mV to -70 mV. The recording electrode was inserted into the center of the contracting muscle to limit the effect of muscle movement on the recording. Signals from all
recordings were amplified and digitized with the use of the MacLab. Chemicals were applied to the preparation directly by hand pipetting 150 uL of chemical to the bath solution of 150 uL.

The action of DEET on the house fly neuromuscular junction was found to be an all or none blockage of the evoked EPSP. Therefore, data was calculated as the percentage of the preparations yielding a blocking response, and was determined from at least five preparations per concentration.

4.2.5.5  Patch clamp recordings

Assistance in patch clamping and associated data analysis was provided by Dr. Baonan Sun. Cells were cultured on cover slips and were immersed in 35 mm Petri dishes filled with room temperature extracellular saline. The recipe for the extracellular patch solution is given in section 4.2.5.1. Patch pipettes were pulled from borosilicate glass capillaries with filament (outer diameter 1.5 mm, inner diameter 0.86 mm (BF150-86-10, Sutter Instrument, Novato CA, USA) on a P-1000 Flaming/Brown micropipette puller (Sutter Instrument). Patch electrodes were filled with solutions containing either intracellular patch solutions described in section.

Cesium (CsCH\(_3\)SO\(_3\)) was used to eliminate potassium currents during sodium channel analysis. The patch electrodes had resistances of 5-7 MOhms. Patch clamp recordings were performed with a 40x/0.80W water immersion objective (working distance 3.5 mm) using a forced-air-cooled Photometrics Evolve 512 with a CCD97 camera system. The neurons were then approached with a patch pipette pressurized to prevent contamination of the tip. After approaching the cell surface, the pressure was released and negative pressure was applied to facilitate seal formation. Following gigaseal formation, the membrane was ruptured through a brief, manual suction of the electrode. If cell rupture could not be obtained after gigaseal formation, a 2 msec long zap pulse was applied.
Currents were amplified with the use of a patch-clamp amplifier (Axopatch 200B, Molecular Devices LLC, Sunnyvale, CA, USA) and were connected via A/D-converter (Digidata 1440A, Molecular Devices LLC). Recordings underwent low-pass filtering at 1 kHz and were sampled at 10 kHz or 100 kHz for potassium and sodium currents, respectively. For recording and analyzing data, pClamp 10.0 software (Molecular Devices LLC) was used. Membrane resistance and capacitance were read from membrane test protocols of pCLAMP 10.0.

4.3 Results

4.3.1 Lethality of DEET

Topical bioassays showed DEET to be toxic via surface contact and topical application (Table 4-1). No statistically significant differences were seen between the mosquito species or strains, indicating no cross-resistance and little species variability. For comparison, the LD_{50} of propoxur to Ae. aegypti is 1 ng/mg, a 1102-fold difference when compared to the LD_{50} of DEET. Synergism with a co-treatment of 200 ng PBO had little to no statistical influence on the topical toxicity of DEET (Table 4-1). The synergism ratio was found to be 1.2-fold for both strains of An. gambiae, 1.02-fold for Ae. aegypti, and 1.3-fold for Musca domestica. DEET was also found to be toxic through contact (e.g. tarsal) exposure. LC_{50} values were found to be much greater than that of propoxur; for Ae. aegypti, there was a 900-fold difference when compared to the LC_{50} of DEET (Table 4-1). DEET was found to be non-toxic to Drosophila melanogaster with a sugar-feeding assay at 1 mg/ml. Higher concentrations were not studied in this assay.

4.3.2 Signs of Intoxication by DEET

The signs of intoxication of Ae. aegypti after exposure to 1750 ng of DEET were found to possess both hyperexcitatory and lethargic tendencies. Five minutes post exposure, the mosquitoes were lethargic, with the majority standing with splayed posture or laying ventral side upward. The mosquitoes were not capable of resting on the vertical sides of the container and
would rest on the flat bottom instead. After agitation, the mosquitoes became hyperexcited through increased wing beat frequency, spinning on their backs, and erratic movements. Flight did not occur even after manual stimulation. After approximately 10 seconds of hyperexcitation, the mosquitoes resumed lethargic tendencies.

The control mosquitoes were found to have normal posture (legs not splayed outward of the midline), would rest on the sides of the cup versus the bottom, and displayed no signs of excitation (twitching, spinning, increased frequency of wing beats, etc). Upon agitation, the mosquitoes would immediately fly from their resting posture to a different location on the container.

Mosquitoes intoxicated with propoxur exhibit extreme hyperexcitation with no lethargic tendencies. Five minutes post-exposure to 1 ng/insect, the mosquitoes were in the supine position and were rapidly beating their wings. This high wing beat frequency caused the mosquitoes to spin on their dorsal side. If the mosquitoes stopped beating their wings, they rapidly twitched and contracted their legs. The mosquitoes continued this behavior until death. Manual agitation of the mosquito caused no change in the behavior of the mosquitoes.

Houseflies presented very similar signs of intoxication in that the flies were lethargic with occasional bouts of excitation. However, unlike Ae. aegypti, the flies did not present a change in behavior after agitation, as they remained standing and did not begin convulsing. Although the flies presented tendencies of excitation by twitching and uncoordinated movements, it was not to the same level of intensity as observed with mosquitoes. The flies were less hyperexcited and more lethargic while intoxicated when compared to the mosquitoes.

4.3.3 Anticholinesterase Actions of DEET and Local Anesthetics

DEET was found to be a poor anticholinesterase inhibitor of DmAChE, hAChE, and MdAChE with mean IC\textsubscript{50} values of 10 mM, 12 mM, and 6 mM, respectively (Table 4-2). The
mosquito enzymes, AgAChE and AeAChE, were found to be completely insensitive to DEET at concentrations up to 10 mM, but were highly sensitive to propoxur, with an IC$_{50}$ value of 447 nM to AgAChE. The mean IC$_{50}$ for propoxur to MdAChE was found to be 130 nM, a 40,769-fold difference when compared to the IC$_{50}$ value of DEET to MdAChE. Lidocaine was found to have an IC$_{50}$ value of 4.9 mM to MdAChE, a 1.1-fold difference when compared to DEET. Toluene was found to be inactive at concentrations up to 10 mM on all enzyme preparations. Chlordimeform was found to be 50-fold more potent (IC$_{50}$: 0.12 mM) to MdAChE when compared to DEET, whereas octopamine had an IC$_{50}$ greater than 10 mM. All IC$_{50}$ values are shown in Table 4-2.

4.3.4 Whole Brain Recordings from Musca domestica larvae

House fly CNS recordings were performed in an effort to further characterize DEET toxicity and to elaborate upon the *in vitro* data. The effects of DEET, lidocaine, and toluene on nerve discharge from larval Musca domestica CNS are shown in Figure 4-3 to display the varying modes of action of the three compounds. DEET was found to be neuroexcitatory to either transected or intact CNS, having EC$_{50}$ values of 0.12 (0.05-0.28) mM and 0.21 (0.08-0.56) mM, respectively. No statistical significance (P > 0.05) was observed between the sensitivity of transected and intact preparations. Toluene was also found to have excitatory properties, but at concentrations ten-fold higher than that of DEET (Figure 4-3). The EC$_{50}$ of toluene was found to be 1.1 mM on the transected CNS. Lidocaine, a known sodium channel blocker, was found to have an inhibitory effect on the CNS with an EC$_{50}$ of 2.4 mM. Propoxur was used as a positive control for AChE inhibitors and was found to have an EC$_{50}$ of 344 nM, 350-fold more potent than DEET. Dose response curves of the three compounds to transected Musca CNS are shown in Figure 4-4.
DEET-mediated neuroexcitation was compared to octopamine to assist in the identification of its mode of action (Figure 4-5). Octopamine was found to have an excitatory EC$_{50}$ of 0.11 mM, nearly identical to the EC$_{50}$ found for DEET (EC$_{50}$: 0.12 mM). Interestingly, chlordimeform, a formamidine insecticide that requires oxidative N-dealkylation to its active principle (Costa and Murphy, 1987), had no effect upon CNS spike discharge frequency. Experiments were then performed with phentolamine, an established octoaminergic antagonist (Yokel and Wise, 1976), to determine its ability to block the neuroexcitation of DEET and test whether neuroexcitation might be mediated via central octopamine receptors. DEET (up to 500 uM) mediated neuroexcitation was completely blocked by 100 µM phentolamine, and it completely blocked octopamine-mediated neuroexcitation at octopamine concentrations up to 3 mM. The neuroexcitation of propoxur was not substantially decreased by phentolamine, as a mere 1.6-fold difference in EC$_{50}$ values were observed with and without phentolamine (Figure 4-5).

### 4.3.5 Sensory Nerve Recordings from *Musca domestica*

DEET was found to have no inhibitory or excitatory influence on the peripheral nervous system of *Musca domestica*. The only change in sensory discharge frequency was gradual rundown in the preparation (Fig. 4-6). Lidocaine was found to inhibit spike frequency at 5 mM, a surprisingly high concentration. Toluene was found to have excitant properties at 5 mM, nearly 10-fold higher than the EC$_{50}$ found in the CNS recordings. The pyrethroid fenfluthrin was used as a positive control and was found to have neuroexcitant properties with an EC$_{50}$ of 25 nM, indicating the assay itself is not the cause of the surprisingly low sensitivity of the sensory system to the three studied compounds. Traces of the nerve recordings are shown in Figure 4-6.
4.3.6 Neuromuscular Junction Recordings from *Musca domestica*

Studies of neuromuscular junctions with DEET yielded a complete nerve block of the evoked EPSP in the body wall musculature of third instar *Musca domestica*. The block was found to be an essentially all or none response with an EC$_{50}$ of 7.2 mM (Figure 4-7). Complete nerve block was obtained approximately 120 seconds after 10 mM DEET was added to the bath, and little graded decline in EPSP amplitude was observed prior to complete block (Figure 4-8). Little to no change was observed on the membrane resting potential after the addition of DEET at any concentration. Lidocaine was also found to be a blocker at the neuromuscular junction and the blocking pattern was consistent with inhibition of presynaptic action potentials, as it was an all or none response very similar to DEET (Figure 4-8). Toluene was found again to be excitatory at 5 mM (data not shown), and the excitatory effect was capable of being washed out after a 14x dilution. Octopamine, a biogenic amine, was found to induce rhythmic discharges at concentrations of 1 mM or greater, but did not induce block of evoked EPSPs (Figure 4-8). Chlordimeform was found to have a graded block of the evoked EPSP at low millimolar concentrations, indicating an effect on the post-synaptic muscle membrane. Propoxur was used as a negative control and was found to have no effect on the NMJ of *M. domestica* larvae (data not shown).

4.3.7 Patch Clamp Recordings

Patch clamp recordings of rat cortical neurons suggested DEET is a blocker of both sodium and potassium channels of rat neuronal cortex cells. DEET was found to block the sodium currents with nearly identical potency when compared to toluene but was 33-fold less active when compared to lidocaine (Figure 4-9a). The DEET-mediated sodium channel block was capable of being washed out to restore approximately 50% of sodium currents (Figure 4-9b).
Potassium channels were more sensitive to DEET as they were blocked at concentrations 8-fold lower than sodium channels (Figure 4-10).

4.4 Discussion

DEET toxicity has been of great interest due to the large number of annual applications to humans. Recent literature suggests that DEET is an acetylcholinesterase inhibitor (Corbel et al., 2009), and suggests that the safety of humans could be at risk through cholinesterase poisoning. Studies were performed to determine the mode of neurotoxic action of DEET and to determine if DEET inhibits acetylcholinesterase at concentrations that imply a reason for concern about its safety. Previous studies (Reuveni and Yagupsky, 1982) have also shown that dermal exposure to DEET can cause redness and numbness of the treated area, similar to an action of local anesthetics. Accordingly, comparisons were made between DEET, lidocaine, and toluene, two documented sodium channel inhibitors to determine the anesthetic properties of DEET (Jaffe and Rowe, 1996; Scior et al., 2009).

DEET was found to be toxic at microgram doses to the mosquitoes, *Ae. aegypti* and *An. gambiae*, and to the housefly, *Musca domestica*. Toxicity assays were performed on the G3 (susceptible) an AKRON (resistant) strains of *An. gambiae* to determine if the G119S point mutation in *AgAChE*, known as MACE, or the upregulated P450 enzyme levels would reduce DEET toxicity. The LD$_{50}$ of the two strains were not statistically significant, indicating that the AChE target site mutation has no relevance to the toxicity of DEET. The addition of pipronyl butoxide had no significant impact on the toxicity of DEET, demonstrating that P450’s are not a primary mechanism of detoxication. To further analyze the potential that DEET is an anticholinesterase, we studied the *in vitro* AChE inhibition of DEET to mosquitoes, houseflies, and human enzymes. Interestingly, we found no inhibition of mosquito AChE and poor inhibition of housefly, *DmAChE*, and human AChE (Figure 4-4). The fact that DEET is toxic to
the mosquito without inhibition of AChE in vitro suggests that DEET imposes its neurotoxic effect in a manner other than acetylcholinesterase inhibition.

A variety of electrophysiological recordings were utilized to further analyze the mode of action of DEET toxicity. The excitation of the CNS after exposure to DEET appeared superficially to be consistent with AChE inhibition but was different than the response pattern of toluene or lidocaine. Lidocaine was found to be a blocker in all preparations studied (e.g. CNS, PNS, NMJ) with EC$_{50}$ values similar to those found in the literature (Jaffe and Rowe, 1996), likely indicating a different action to nerve preparations when compared to DEET, regardless of the similar $Md$AChE inhibition potencies seen in Table 4-2. Although DEET and lidocaine were nearly equipotent against $Md$AChE, the local anesthetic action of lidocaine on presynaptic sodium channels likely negates the anticholinesterase action. Toluene was uniformly excitatory in all preparations with no anesthetic action on the insect nervous system. This suggests the insect local anesthetic binding site (LABS) of the sodium channel is different when compared to rat LABS (Scior et al., 2009).

The insect neuromuscular junction serves as a negative control for effects of suspected cholinergic inhibitors, as insects do not have cholinergic synapses in their peripheral nervous system (Calhoun, 1963; Booth and Lee, 1971). Within this preparation, DEET was shown to exhibit presynaptic block of the evoked EPSP, an effect consistent with an anesthetic-like action. Lidocaine displayed a blocking pattern similar to DEET, indicating an effect on the presynaptic nerve terminal. This similar mode of action on the neuromuscular junction is likely due to the similar chemical structures these two compounds possess. As expected, propoxur was found to be inactive on the $M.~domestica$ neuromuscular junction and served as a negative control. Because DEET has effects inconsistent with cholinesterase inhibition and an action different than
lidocaine based on the CNS studies, experiments were performed to compare DEET to compounds acting on the octopmaninergic system.

Octoapaminergic compounds, such as chlordimeform (CDM) and octopamine, are toxic through an activation of octopamine receptors, leading to an increased cAMP concentration and overstimulation of the octopaminergic synapse. In addition, chlordimeform is documented to possess both anesthetic and excitatory effects as the chemical blocks nerve transmission at high concentrations, but can have an excitatory effect at lower concentrations (Pfister et al., 1978; Hollingsworth and Lund, 1982). Further comparison between DEET and octopaminergic compounds found that phentolamine completely blocked the CNS neuroexcitation of DEET and octopamine, but was not found to be an effective blocker of propoxur. These data suggest DEET is likely targeting octopaminergic synapses and not acetylcholinesterase to induce toxicity.

The structure of DEET superficially resembles the chemical structure of lidocaine and chlordimeform (Fig. 4-1), a characteristic that should not be overlooked when studying the potential for a similar mode of action. The largest stuctural difference between DEET and lidocaine is the basicity of the lidocaine nitrogen, and for DEET and chlordimeform would be the presence of a chlorine atom substituted at the 4 position on the phenyl ring. Both chemicals possess a substitutd side chain with DEET having a carbonyl amide and one diethylamine group while chlordimeform possesses the formamidine dimethyl groups and no carbonyl oxygen. Three-dimensional structural overlays (Figure 4-11) of the two chemicals suggest the rings overlap well, and the diethylamine group of DEET can occupy space similar to the dimethylamine of CDM. These similar structural bases could provide similarities in their action on ion channels and enzymatic systems.
Patch clamp studies were performed with rat neuronal cortex cells to determine the mode of action of DEET on specific ion channels within mammalian cells. Results indicate DEET is capable of blocking sodium and potassium channels at low to mid micromolar concentrations, similar to lidocaine and other local anesthetics. These results can be useful for describing the numbing sensation observed when DEET contacts mammalian skin (Reuveni and Yagupsky, 1982). With regards to insect toxicity, it is unlikely sodium or potassium channel blockage is the primary mechanism of toxicity. Lidocaine and CDM have been documented to have anesthetic-like effects through noncompetitive inhibition of acetylcholine and inhibition of potassium induced contractions (Wang et al., 1975). Although DEET is capable of blocking sodium channels at similar concentrations to known anesthetics (lidocaine and toluene), it is unlikely DEET is capable of decreasing the synaptic sensitivity to acetylcholine leads to insect toxicity. We believe this is unlikely based on the data showing that DEET is a neuroexcitant of the CNS, whereas a block of spike frequency was observed with lidocaine. Mosquitoes show a splayed posture and hyperexcitation after toxic doses of DEET, perhaps because of a combination of central octopaminergic hyperexcitation complexed with incomplete suppression of peripheral neurotransmission.

To conclude, it is unlikely that DEET exerts its toxicity through anticholinesterase properties due to its low potency for enzyme inhibition, and the block of neuroexcitation with phentolamine. Thus, it is plausible to suggest toxicity to houseflies, and likely mosquitoes, is through a mimicking action of the neurotransmitter octopamine in the octopaminergic system as was portrayed with the CNS recordings performed with phentolamine, perhaps in combination with peripheral neurosuppressive effects. Numbness of mammalian mucous membranes is potentially explained through an anesthetic effect of nerve conduction block.
Figure 4-1. Structures of inhibitors used in Chapter 4.
Figure 4-2. Diagram of the *Musca domestica* dissection, recording arrangement, and nerve activity for the central nervous system (A) and the sensory nervous system (B) dissections.
Table 4-1. Toxicity values of DEET to three mosquito strains and the house fly. Letters after 95% CI values represent statistical significance for IC$_{50}$ values among species within a given toxicity measurement column (first letter) and between synergized and unsynergized LD$_{50}$ for each strain (second letter). Values not labeled by the same letter represent statistical significance at P < 0.05.

<table>
<thead>
<tr>
<th>Species</th>
<th>LC$_{50}$ (mg/ml; 95% CI)</th>
<th>LD$_{50}$ (ng/mg; 95% CI)</th>
<th>LD$_{50}$ (+ 200 ng PBO) (ng/mg; 95% CI)</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag (G3)</td>
<td>1.9 (0.9-2.9)a</td>
<td>1175 (988-1361)a,a</td>
<td>1021 (829-1231)a</td>
<td>1.2</td>
</tr>
<tr>
<td>Ag (AKRON)</td>
<td>2.7 (1.0-4.5)a</td>
<td>1472 (1136-1806)b,a</td>
<td>1247 (872-1526)a</td>
<td>1.2</td>
</tr>
<tr>
<td>Ae. aegypti</td>
<td>2.3 (1.1-3.6)a</td>
<td>1102 (836-1367)a,a</td>
<td>1082 (841-1310)a</td>
<td>1</td>
</tr>
<tr>
<td>M. domestica</td>
<td>-</td>
<td>8104 (7026-9000)-,a</td>
<td>6219 (4405-8563)a</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Table 4-2. Enzyme inhibition data expressed as mean (n=3) IC\(_{50}\) values.

<table>
<thead>
<tr>
<th></th>
<th>IC(_{50}) (mM; 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Propoxur</td>
</tr>
<tr>
<td><strong>An. gambiae (G3)</strong></td>
<td>4.5e-4 (4.2e-4 - 4.8e-4)</td>
</tr>
<tr>
<td><strong>An. gambiae (AKRON)</strong></td>
<td>&gt; 1</td>
</tr>
<tr>
<td><strong>Ae. aegypti</strong></td>
<td>3.7 e-4 (3.2e-4 - 4.1e-4)</td>
</tr>
<tr>
<td><strong>M. domestica</strong></td>
<td>1.3e-4 (1.1e-4 - 1.6e-4)</td>
</tr>
<tr>
<td><strong>Drosophila melanogaster</strong></td>
<td>8.4e-5 (6.9e-5 - 1.1e-4)</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td>1.4e-3 (1.2e-3 - 1.6e-3)</td>
</tr>
</tbody>
</table>
Figure 4-3. Nerve discharges of the CNS from *M. domestica* third instar larvae.
Figure 4-4. Potency of DEET, toluene, and lidocaine on CNS nerve discharge of the house fly

- **DEET**
  - EC$_{50}$: 0.12 mM (0.05–0.3)

- **Toluene**
  - EC$_{50}$: 1.1 mM (0.8–1.4)

- **Lidocaine**
  - EC$_{50}$: 0.24 mM (0.2-0.4)
Figure 4-5. Effect of phentolamine on the activity of DEET, octopamine, and propoxur to CNS nerve discharge rates of the housefly.
Figure 4-6. Sensory nervous system firing frequency recordings from *M. domestica* third instar larvae.
Figure 4-7. Concentration-response relationship for the blocking action of DEET at the neuromuscular junction of Musca domestica. Numbers next to data points represent the number of preparations studied at each concentration.
Figure 4-8. Recordings of evoked EPSP of the neuromuscular junction in *M. domestica* larvae after exposure to DEET, lidocaine, chlordimeform (CDM), and octopamine. In the DEET trace, the remaining transients after block of the EPSP are stimulus artifacts. Expansion of the octopamine trace (arrow) shows the rhythmic activity induced by high concentrations of octopamine.
Figure 4-9. Voltage dependent block of rat neuronal sodium channels. A) Dose response curves of three inhibitors to rat neuronal sodium channels obtained through patch clamp recordings. B) DEET mediated voltage dependence block of the sodium currents in rat neuronal cortex neurons.
Figure 4-10. Voltage dependent block of rat neuronal potassium channels. A) Dose response curves of DEET mediated inhibition of rat neuronal potassium channels obtained through patch clamp recordings. B) DEET mediated voltage dependence block of the potassium currents in rat neuronal cortex, expressed as current-voltage plots.
Figure 4-11. Three-dimensional structural overlay of the carbon backbone of DEET (green) and chlordimeform (magenta). Other atoms are carbonyl oxygen (red), nitrogen (blue), and hydrogen (white). Model was created by Dr. Dawn Wong using 3DpyMol.
CHAPTER 5
ACTIVITY OF NEWLY DESIGNED ANOPHELES GAMBIAE SELECTIVE CARBAMATES AGAINST MOSQUITO VECTORS, AGRICULTURAL PESTS, AND MODEL ORGANISMS

Abstract: New and highly selective anticholinesterase mosquitocides (ie: carbamates) are under development in our laboratory. The experimental carbamates have shown up to 1000-fold selectivity of An. gambiae enzyme over human AChE and an LD$_{50}$ of 4 ng/insect. A similar degree of selectivity and toxicity was observed for other mosquito species studied. For example, the compound, 3-tert-butylphenyl-N-methylcarbamate yielded: An. gambiae IC$_{50}$ ≤ 104 nM and an LD$_{50}$ of 4 ng/mosquito; Ae. aegypti IC$_{50}$ ≤ 79 nM and LD$_{50}$ ~ 7 ng/mosquito; Cx. quinquefasciatus IC$_{50}$ ≤ 120 nM and LD$_{50}$ ~14 ng/mosquito. Surprisingly, the experimental carbamates displayed generally poor enzyme inhibition in the honey bee (Apis mellifera) and lepidopteran agricultural pests (IC$_{50}$ > $10^{-6}$), indicating unusual insect selectivity.

The unique selectivity pattern of our experimental carbamates has implications for mitigation of mosquito insecticide resistance due to agricultural uses. It has been suggested that irrigated agriculture and crop spraying has subjected mosquito vectors to selection in the larval stages, especially with pyrethroids. Development of insecticides that have little to no toxicity on agricultural pests, such as our novel carbamates, can alleviate this mode of insecticide resistance selection by eliminating agricultural use.

5.1 Introduction

The use of synthetic chemicals remains the principal mechanism of integrated vector management for the control of malaria and other vector borne diseases (Hemingway and Ranson, 2000). Residents of malaria endemic countries sleep under insecticide treated nets (ITNs) to reduce malarial transmission. Pyrethroids remain the only class of insecticides approved for use in insecticide treated nets (ITNs), the first line of malaria vector control, and have been effective
in controlling the malaria vector for a number of years. However, the increased prevalence of pyrethroid-resistant mosquitoes, primarily through a sodium channel mutation (kdr), is forcing researchers to develop new mosquitocides with a novel mode of action to control the malaria mosquito, *An. gambiae*. Besides malaria, diseases such as dengue fever/dengue hemorrhagic fever (vector: *Ae. aegypti*), lymphatic filariasis (vector: *Cx. quinquefasciatus*), and West Nile Virus (vector: *Ae. albopictus*) are also highly prevalent and often deadly. By 1999, the WHO deemed forty percent of the world’s population to be at risk for dengue fever and over a billion individuals, in as many as eighty countries were at risk for lymphatic filariasis (WHO, 2007). It is apparent that the design of novel mosquitocides is vital for the reduction of vector-borne disease transmission and minimizing mosquito-borne deaths.

Acetylcholinesterase (AChE) is a well-validated insecticide target site that has been utilized for many years with organophosphates and carbamates. AChE is a serine hydrolase that is necessary for regulation of the neurotransmitter acetylcholine in both, humans and insect central nervous systems. Anticholinesterases react with a serine residue located at the catalytic site near the bottom of the AChE gorge to inactivate the enzyme. The inactivated enzyme is no longer capable of hydrolyzing acetylcholine, resulting in the buildup of Ach in the nerve synapse, leading to death (O’Brien, 1967; Pang, 2006). Design of novel mosquitocides that possess high levels of mosquito selectivity could have large implications for vector disease control programs.

Insecticide resistance of mosquitoes due to agricultural uses has been documented and specifically affects insecticide design for disease control. Widespread agricultural use of pyrethroids has been implicated in exacerbating development of resistance to insecticides with the same mode of action, thus reducing the effectiveness of ITNs (Yadouleton et al., 2009).
Development of a highly selective insecticide with poor toxicity to agricultural pests can mitigate resistance due to less ancillary uses and therefore, limited selection pressure within breeding sites.

Our novel carbamates possess a high degree of selectivity through utilization of unique differences between human and *An. gambiae* AChE active sites. The objective of this research was to determine the *in vitro* and *in vivo* activity of experimental insecticides to other nuisance biting mosquitoes, model organisms, and agricultural pests in an effort to gain a broader perspective on the potential toxicity of these compounds to other insects.

5.2 Materials and Methods

5.2.1 Inhibitors, Solvents, and Assay Reagents

Propoxur (99% purity), bendiocarb (99% purity), and carbofuran (99% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Experimental carbamates were prepared as described in Carlier et al., 2008. All experimental compounds were purified by column chromatography and/or re-crystallization and are >95% pure by $^1$H NMR analysis. Structures of experimental carbamates and propoxur are shown in Figure 5-1.

Ellman assay (Ellman et al., 1961) reagents are composed of acetylthiocholine iodide (ATCh) ($\geq 99\%$ purity), 5,5'-dithiobis-(2-nitro)benzoic acid (DTNB) (99% purity), and sodium phosphate buffer, all of which were purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular sieve OP type 3Å were purchased from Sigma (St. Louis, MO, USA) and were used to prevent water absorption within the DMSO stock. Fifty beads were added into a 100 mL stock solution. These sieves have a diameter of \( \sim 2 \text{ mm} \), a pore size of 3Å, and a water absorbing capacity of $\geq 15\%$. The solvents, dimethyl sulfoxide and absolute ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA).
5.2.2 Insects and Enzyme sources

Wildtype *An. gambiae* (AgAChE) (Diptera: Culicidae) mosquitoes were provided by the Center for Disease Control (Atlanta, GA) and were reared from the egg lifestage at the University of Florida (Department of Entomology and Nematology, Emerging Pathogens Institute, Gainesville, FL, USA). *An. albimanus* (AaAChE) (Diptera: Culicidae), *An. quadrimagulatus* (AqAChE) (Diptera: Culicidae), and *Ae. aegypti* (AeAChE) (Diptera: Culicidae) were cultured and reared at the United States Department of Agriculture – Agricultural Research Service (Gainesville, FL, USA). *Ae. albopictus* (AbAChE) (Diptera: Culicidae) is a non-resistant lab strain that was provided by Dr. Phil Kaufman at the University of Florida (Department of Entomology, Medical and Veterinary Laboratory, Gainesville, FL, USA). *Cx. quinquerecussiatus* (CqAChE) was supplied by Dr. Bill Walton at the University of California, Riverside and is a 40-year, susceptible lab strain and was used for toxicity assays. The housefly, *Musca domestica* (FS strain) (Diptera: Muscidae), was provided by Dr. Phil Kaufman at the University of Florida (Department of Entomology, Medical and Veterinary Laboratory, Gainesville, FL, USA) and has been in culture for 40 years. *Drosophila melanogaster* (Orgeon-R strain) (Diptera: Drosophilidae) was cultured at the University of Florida (Department of Entomology and Nematology, Emerging Pathogens Institute, Gainesville, FL, USA). Asian Citrus Psyllids, *Diaphoria citri* (Hemiptera: Psyllidae) were provided by the Department of Entomology and Nematology, Lake Alfred CREC station, at the University of Florida (Gainesville, FL, USA). The honeybee, *Apis mellifera* (AmAChE) (Hymenoptera: Apidae), was provided by Dr. James Ellis at University of Florida (Department of Entomology, Bee Unit, Gainesville, FL, USA). *Ostrinia nubilalis* (Lepidoptera: Crambidae) were ordered from French Agricultural Research (Lamberton, MN). *Plutella xylostella* (Lepidoptera: Plutellidae) was provided by Dr. Tony Shelton at Cornell University (Ithaca, NY, USA). Neither
lepidopteran species are known to possess resistance to any insecticides. Human AChE enzyme was purchased from Sigma-Aldrich (lyophilized powder, Sigma C1682, St. Louis, MO, USA).

Acetylcholinesterase enzyme sources were prepared from groups of ten whole non-blood fed adult female mosquitoes, three fly/bee heads, six whole bodied fruit flies, twenty whole bodied psyllids, or twenty L3 lepidopteran heads. Each enzyme preparation was from tissue homogenized in 1 mL of ice-cold sodium phosphate buffer (0.1 M, pH 7.8) containing 0.3% Triton x-100, with an electric motor driven glass tissue homogenizer. The homogenate was centrifuged at 5000 x g at 4°C for 5 minutes. The supernatant was used as the enzyme source for the assay. Prior to use in assay, hAChE was diluted 100x with the aforementioned buffer + Triton mixture.

5.2.3 Enzyme Inhibition Assays

IC$_{50}$ values (concentration needed to inhibit 50% of the enzyme) were determined using slight modifications from Ellman et al. (1961) and is outlined in Carlier et al. (2008). Briefly, 10 μL of enzyme solution was added to each well of the 96-well micro assay plate along with 20 μL of dissolved compound and 150 μL of ice-cold phosphate buffer. The assay plate was incubated at 25°C for ten minutes. Ellman assay reagents, ATCh (0.4 mM, final conc.) and DTNB (0.3 mM, final conc.), were prepared new for each experiment and 20 μL was added to the enzyme to initiate the reaction. Changes in absorbance were recorded by a DYNEX Triad spectrophotometer (DYNEX Technologies, Chantilly, VA, USA) at 405 nm. Six inhibitor concentrations were used in triplicate to construct dose-response curves using Graphpad Prism 4 (GraphPad Software, San Diego, CA, USA). Inhibitors were prepared using DMSO and contained a final concentration of 0.1% DMSO (v/v) throughout each inhibitor concentration. Enzyme concentrations used were within the linear range, therefore eliminating the need for
protein quantification. IC$_{50}$ values for each species were calculated by nonlinear regression from eight inhibitor concentrations using Prism$^{TM}$ (GraphPad Software, San Diego, CA, USA). The nonlinear regression equation used was as follows:

$$Y = \text{bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{((\log\text{EC}_{50} - x) \times \text{Hillslope})}};$$

where $x$ = the logarithm of the concentration and $Y$ = the response.

$Y$ start at the top (normalized 100%) and approaches the bottom (0%) with a sigmoid shape.

5.2.4 Topical Toxicity Assays

Topical toxicity bioassays were performed based on the method of Pridgeon et al (2008). Briefly, insects were chilled on ice for 3 min, during which the appropriate volume (200 nL for mosquitoes, 1 μL for lepidopteran larvae) of chemical (dissolved in 95% ethanol) was applied onto the abdomen of the insect using a handheld Hamilton® microapplicator. For each inhibitor, five doses were applied to ten insects each, and repeated three times. An ethanol-only treatment was included in each experiment as a negative control. Insects were transferred into paper cups covered with netting and supplied with free access to sugar water for the duration of the experiment. Mortality was recorded at the 24-hour time point. Mortality data was pooled and analyzed by log-probit using Poloplus® to determine 24 hr LD$_{50}$ values. Three LD$_{50}$ values were obtained and the mean LD$_{50}$ value was used for statistical analysis.

5.2.5 Mouse Oral Toxicity

Mammalian toxicity studies provide valuable insights toward structure activity relationships and safety determinations of novel inhibitors. We performed a screen of three experimental insecticides using the OECD/OCDE approved method, known as the “up-and-down method” (ASTM, 1987; Dixon and Mood, 1948; Bruce 1985), in oral dosing of male $M_{us}$
musculus (ICR strain). All procedures for these experiments were approved by the University of Florida IACUC.

Inhibitors were dissolved in a 10% DMSO-olive oil mixture and the final concentration of DMSO was 0.1% for each inhibitor concentration. Drugs were administered through the use of an oral gavage needle at volumes of no more than 400 uL. A maximum of eight mice total were used for each inhibitor and were monitored every 4 hours for 24 hours after the administration of the insecticide. Toxicity was recorded at 24 hours post exposure. The mice were sacrificed at any sign of suffering and counted as dead.

5.2.6 Statistical Analyses

The IC\textsubscript{50} and Hill slope values for carbamates run under each protocol were averaged (n=3) and compared by a one-way ANOVA followed by Tukey’s multiple comparison test using GraphPad InStat\textsuperscript{TM} (GraphPad Software, San Diego, CA, USA). Mortality was recorded 24 hours post treatment and an LD\textsubscript{50} was calculated using Poloplus\textsuperscript{®}. Three LD\textsubscript{50} values were obtained and the mean LD\textsubscript{50} value was used for statistical analysis.

For all toxicity assays, control mortality was corrected for using Abbots formula (Abbot, 1925).

Abbotts Formula: Corrected percent mortality = (\% alive in control - \% alive in treated) / \% alive in control

5.3 Results

5.3.1 Pharmacodynamic Studies

5.3.1.1 Mosquito vectors

Standard and experimental methylcarbamates were screened on five mosquito species prior to screening on agricultural pests and model organisms. All data were fit to a sigmoid curve with \( r^2 \geq 0.98 \) within all experiments and Hill slope values \( \geq 0.8 \). The IC\textsubscript{50} results of
mosquito enzymes, presented in Table 5-1, show that carbofuran displayed more potent inhibition across all mosquito species than all other methylcarbamates. Carbofuran was found to be approximately 10-fold more potent than the least potent commercial carbamate, propoxur. In addition, the rank order of potency of commercial carbamates insecticides was always carbofuran > bendiocarb > propoxur (Table 5-1). No statistical significance of the IC₅₀ value was observed between mosquito species for each separate commercial carbamate.

The potencies of experimental carbamates were widely variable among the mosquito species tested (Table 5-1). PRC 331 and PRC 388 were the two experimental inhibitors studied that contain a meta substituted side chain. Of these two, PRC 331 was found to be the more potent by approximately two-fold. PRC 408 was found to be the most potent experimental inhibitor containing an ortho substituted side chain in all mosquito species studied. Differences ranged from 1.3-fold (AeAChE; PRC 408 and PRC 337) to 6.1-fold (AgAChE; PRC 408 and PRC 421) for ortho substituted experimental carbamates on mosquito species. Of the Anopheles spp. studied, PRC 331 and PRC 408 displayed nearly identical inhibition potencies (ca. 100 nM), however, PRC 408 was approximately two-fold less potent when compared to PRC 331 in both Ae. mosquito species (Table 5-1).

5.3.1.2 Agricultural pests

All IC₅₀ values of agriculturally relevant insects are shown in Table 5-2. Three agricultural pests and one economically important pollinator were studied to determine the activity of experimental inhibitors to agriculturally relevant insects. Lepidopteran insects, Plutella xylostella and Ostrinia nubilalis, were significantly less sensitive to the experimental carbamates when compared to An. gambiae. When compared to AgAChE, O. nubilalis displayed up to a 194-fold increase (PRC 408) in IC₅₀ value, whereas the commercial carbamate bendiocarb displayed a 1.9-fold increase. The pattern of decreased inhibition potencies was also
observed with *Plutella xyostella* AChE as experimental inhibitors displayed up to a 492-fold decrease (PRC 408) in inhibition, whereas bendiocarb increased 1.2-fold, a statistically insignificant increase between AgAChE and PxAChE. Inhibition potencies also varied widely with *D. citri* as there was a ten-fold difference between the two commercial carbamates, propoxur and bendiocarb. For the experimental insecticides, PRC 331 was the most potent inhibitor (128 nM) and was two-fold more potent than PRC 388, the other meta substituted experimental methylcarbamate. The experimental carbamates possessing an ortho substituted side chain were 8- to 48-fold less active to *D. citri* when compared to PRC 331 (Table 5-2).

The economically important pollinator, *Apis mellifera*, displayed a wide range of inhibition potencies that appear to be based upon the position of the substituted side chain. Similar to *D. citri*, all ortho substituted inhibitors were substantially less potent when compared to meta substituted inhibitors. A 19-fold difference in inhibition was observed between propoxur and bendiocarb, whereas a 170-fold difference in inhibition was observed between PRC 331 and PRC 421, the most and least potent experimental inhibitors. Of the experimental carbamates, a 2.4-fold difference was observed between meta substituted compounds and up to a 14-fold difference was observed between the ortho substituted compounds (Table 5-2).

### 5.3.1.3 Model organisms

The flies, *Drosophila melanogaster* and *Musca domestica*, were shown to possess similar inhibition potencies to both standard and experimental carbamates (Table 5-3). The most potent commercial inhibitor was bendiocarb, which was 2.6-fold (*MdAChE*) to 3.8-fold (*DmAChE*) more potent when compared to propoxur. The experimental inhibitor PRC 408 was the most potent inhibitor for both fly species with inhibition values 23- to 13- fold more active than PRC 421 for *DmAChE* and *MdAChE*, respectively (Table 5-3). Human AChE was found to be much less sensitive to the experimental carbamates when compared to both fly and mosquito species.
PRC 331 and PRC 421 were found to be the most and least potent inhibitors, respectively, for human AChE. *Torpedo californica* AChE inhibition values were obtained from Jiang et al. (submitted, 2012) and are included in this dissertation as a model fish species. *TcAChE* inhibition values suggest the enzyme is much more sensitive to *meta* substituted inhibitors as the IC$_{50}$ values of these compounds range from 167 nM to 221 nM, a 1.3 fold difference. However, PRC 421, an *ortho* substituted carbamate, is 44-fold less potent when compared to PRC 331 (Table 5-3).

### 5.3.2 Human Selectivity and Mammalian Toxicity of Experimental Carbamates

Data show that the experimental carbamates possess selectivity values (human IC$_{50}$/AgAChE IC$_{50}$) of up to 261, an 87-fold increase in selectivity when compared to propoxur (Table 5-3). The experimental carbamates, PRC 331 and PRC 388 had the lowest SR values, no more than 2-fold. The *ortho*-substituted experimental carbamates were found to be substantially more selective with SR values ranging from 16- to 261-fold over human AChE (Table 5-3). These selectivity ratios were also reported in Jiang et al., (submitted, 2012).

Mouse toxicity data support the *in vitro* results, as the experimental inhibitors were at least 10-fold less toxic when compared to propoxur. The least toxic inhibitors to the mouse was PRC 408 and PRC 421 with LD$_{50}$’s of 1700 mg/kg and >2000 mg/kg, respectively.

### 5.3.3 Selectivity of Experimental Carbamates Over Agricultural Insects

Selectivity ratios are used to express *in vitro* selectivity differences between *An. gambiae* AChE, the enzyme for which the experimental carbamates were designed, and various agricultural insect AChEs, as shown in Table 5-4. Selectivity ratios (SR) were compared to *An. gambiae* IC$_{50}$ values since the experimental carbamates were designed to control this particular species of mosquito. Selectivity ratios of commercial carbamates were found to be 1- to 2-fold for *D. citri* and *A. mellifera*, yet ranged from 2- to 15-fold selective for *O. nubilalis*. Propoxur
was found to be the most selective commercial carbamate (15-fold) for *O. nubilalis*, but was 13-fold less selective than PRC 408, the most selective experimental carbamate (Table 5-4). PRC 408 was found to be 97-fold more selective than bendiocarb. Commercial carbanates were found to be poorly selective over the AgAChE enzyme for *D. citri* and selectivity of experimental carbanates were found to range from 0.9-fold (PRC 388) to 30-fold (PRC 408). PRC 408 was found to be 15-fold more selective than either commercial carbamate studied (Table 5-4).

Commercial carbanates bendiocarb and propoxur were found to be 1- to 2-fold selective over AgAChE for AmAChE. Experimental carbanates displayed SR values over AgAChE ranging from 0.3-fold (PRC 331) to 12-fold (PRC421), and PRC 331, 337 and 388 were negatively selective for AmAChE. However, PRC 408 and PRC 421 were up to 11- and 12-fold more selective than the commercial carbamate bendiocarb, respectively (Table 5-4).

### 5.3.4 Toxicity of Methylcarbanates

Toxicity of carbanates was assessed through topical bioassays to determine the LD$_{50}$ values (Table 5.5). Experimental carbanates were found to range in LD$_{50}$ 20-fold, with PRC 331 and PRC 421 being the most and least toxic to *An. gambiae*, respectively. These data support the *in vitro* data as they were the most and least potent inhibitors, as well (Table 5-5). The two commercial carbanates were found to have an LD$_{50}$ of 3 ng/insect (propoxur) and 2 ng/insect (bendiocarb) to *An. gambiae* adults. The experimental inhibitor, PRC 331, was found to be nearly equitoxic when compared to the two commercial carbanates. Excluding PRC 421, all other experimental carbanates were considered to be highly toxic to *An. gambiae* with LD$_{50}$ values $\leq$ 12 ng/insect. Experimental inhibitors were found to be slightly less toxic to *Ae. aegypti* when compared to *An. gambiae*, as LD$_{50}$ values ranged from a 1.4- to 18-fold difference when compared to propoxur (LD$_{50}$: 5 ng/insect). PRC 331 was found to be nearly equitoxic to *Ae.*
When compared to propoxur, and was 3-fold less toxic when compared to bendiocarb. As with Ag, excluding PRC 421, LD₅₀ values were found to be ≤ 23 ng/insect and were deemed to be highly toxic to Ae aegypti. Toxicity of methylcarbamates to Cu. quinquefasciatus was found to be substantially less with commercial and experimental carbamates by 3- to 11- fold when compared to An. gambiae. However, the least toxic carbamate, PRC 421 was found to be nearly equitoxic (≤ 1.1-fold difference) to all three mosquito species. Interestingly, the experimental carbamates possessed low toxicity to Ostrinia nubilalis. Bendiocarb was found to have an LD₅₀ of 101 ng/insect, 23-fold more toxic than the most toxic experimental carbamate (PRC 331). Experimental carbamates and propoxur were all toxic at low microgram doses to Ostrinia. These data correlate with the poor potency activity that was observed during the in vitro analysis.

5.4 Discussion

The currently registered carbamates (eg. propoxur, bendiocarb, carbofuran, etc) could be improved upon due to the minimal selectivity the compounds possess. To compound the issue, the increasing prevelance of pyrethroid resistant mosquitoes has increased the need for the design of chemicals with a novel mode of action to augment current control methods. Similarly, mosquito control in South Florida relies on ultra low volume spraying of organophosphates to control Ae. aegypti and the spread of dengue fever. Insecticide resistance and the incidental toxicity to endangered insects, has limited the uses of currently utilized insecticides, amplifying the need for the development of novel chemicals.

All mosquito species studied were highly sensitive to the experimental carbamates (Table 5-1), and most novel carbamates were found to be toxic at levels equal or near that of propoxur (Table 5-5). Literature suggests that alkyl substituents at the meta- position of the phenyl ring are more potent inhibitors than substitutions at the ortho- position (Metcalf, 1971; Kuhr and
Dourough, 1976). Although this was true for the agriculturally relevant insects, this trend was not observed for the potency of mosquitoes as PRC 331 and PRC 408 were nearly equipotent in all five mosquito species studied. Among the ortho-substituted carbamates, propoxur and PRC 421 both possess an alkoxy linkage to the phenyl ring while PRC 337 and PRC 408 are thioethers (Fig. 5-1). The structural similarities between PRC 421 and propoxur likely explain the similar inhibition potencies observed across all mosquito species. However, it is interesting to note that although both are thioethers substituted in the ortho-position, PRC 408 is approximately four-fold more potent than PRC 337 across all mosquito species. This reduction of activity suggests the double bond in PRC 337 prevents flexibility, making the enzyme less accommodating to the rigid structure. The high activity observed with PRC 408 was reduced four- to five-fold by replacing the sulfur with an oxygen (PRC 421). Similarly, meta-substitution of a silicon group in the side chain of PRC 388 causes a reduction in potency when compared to the t-butyl group of PRC 331, suggesting the larger silicon group reduces the access to the catalytic site in all mosquito species.

Selectivity against human AChE is of critical importance for development of insecticides with the intended use being ITN deployment. Mouse toxicity data for propoxur, a WHO approved carbamate for mosquito control, has a mammalian LD_{50} of 24 mg/kg (Black et al., 1973) and is ten-fold more toxic when compared to PRC 331 (254 mg/kg) to mice. Branching schemes containing a sulfur or oxygen atom off the ortho position, as seen in PRC 408 and PRC 421, decreases the mammalian toxicity by up to 75-fold when compared to Propoxur. These data suggest the novel carbamates are substantially safer than the currently utilized methylcarbamates for malaria control. Previous studies (Hartsel et al., 2012) have shown models suggesting AgAChE has a larger ligand pocket when compared to hAChE. This larger binding pocket of
AgAChE likely accepts inhibitors with an increased steric bulk approximately two bonds away from the phenyl core with a greater affinity when compared to hAChE, yielding selectivity. Therefore, the larger 2-substituted side chains (PRC 388) have a reduced mammalian selectivity when compared to the smaller 2-substituted side chain in PRC 331. Similarly, the longer and more rigid ortho-substituted side chains have reduced SR values when compared to the more flexible, and somewhat shorter, ortho-substituted side chains (Hartsel et al., 2012; Jiang et al., submitted).

Although the novel carbamates were found to possess high activity against mosquito species and low activity against mammalian models, it is also critical to understand the activity of the chemicals to agricultural pests and non-target organisms. Broad spectrum insecticides were once favored for commercialization due to the ability to target numerous pests with the same chemical. However, insecticide resistance of mosquitoes due to agricultural uses has been documented, and specifically affects insecticide design for disease control. Widespread agricultural use of pyrethroids has been implicated in exacerbating development of resistance to insecticides with the same mode of action when used in ITNs (Yadouleton et al., 2009). Currently, lepidopteran insect pests are considered to be the most important insect pest of maize in Africa, and are the cause of substantial food loss throughout the continent (Ong’Amo et al., 2006). Specifically, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) is a lepidopteran stem borer known to cause large amounts of economic damage in Eastern Africa (Ong’Amo et al., 2006). The European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), was studied due to its relatedness to *Chilo partellus* in an effort to determine the activity of novel carbamates to lepidopteran pests. Results show high selectivity for AgAChE over lepidopteran AChE enzyme
with all novel carbamates (Table 5-4), suggesting advantageous properties for mitigation of insecticide resistance through reducing the ancillary uses of the chemicals in crop pest control.

The novel carbamates with meta- positioned side chains were inversely selective against *D. citri* and *A. mellifera*, whereas the ortho- substituted carbamates were up to 30-fold selective. Additionally, propoxur, a commercialized ortho substituted carbamate, and the ortho substituted experimental inhibitors were found to be poor inhibitors of *D. citri* and *A. mellifera*, which likely indicates these proteins are less accommodating to alkane substituted side chains in the ortho position versus the meta position. These data suggest ortho-substituted, branched carbamates comprise a promising approach to more selective carbamates against *An. gambiae* based upon the high mosquito activity and poor agricultural insect activity.

Control of *Ae. aegypti* in the state of Florida is becoming increasingly difficult due to insecticide resistance, and the large abundance and diversity of environmentally sensitive lands found within the Florida complicate the problem of controlling mosquito vectors (Zhong et al., 2010; Zhong, 2007). One organism of interest is *Cyclargus thomasi bethunebakeri*, or the Miami Blue Butterfly (MBB). The MBB is a coastal butterfly that inhabits sunny areas at the edge of tropical hardwood forests (Zhong, 2007). This species of insect is considered to be one of the rarest insects in North America, and was declared to be endangered by the state of Florida in 2002. Currently, Bahia Honda Key State Park is the only isolated population of MBB that remains flourishing in the wild (Center of Biodiversity, 2005). Due to this being the only wild population remaining, there are large controversies over utilizing insecticides for dengue control near potential MBB habitats. It is plausible to suggest utilization of the novel carbamates discussed in this study could be implemented for successful dengue control while allowing the population of the endangered butterfly species to grow in the presence of the insecticides.
In conclusion, the potent AChE inhibition of mosquito AChE and previously undocumented levels of high mouse oral toxicity coupled with the low activity to agricultural insects suggest these are likely candidate insecticides to be employed into any mosquito control program. The general lack of activity against agricultural pests suggests avoidance of cross resistance development in non-target species from incidental insecticide exposure from agricultural uses.
Figure 5-1. Chemical structures of methylcarbamates tested for activity to mosquito vectors and agriculturally relevant insects. Bendiocarb and carbofuran are not shown due to their presence in the literature and unrelated characteristics when compared to the experimental carbamates.
Table 5-1. Mean (n=3) inhibition potencies of commercial and experimental methylcarbamates to five mosquito species in the genera *Anopheles* and *Aedes*. Abbreviations are as follows: AgAChE (*Anopheles gambiae*), AaAChE (*Anopheles albimanus*), AqAChE (*Anopheles quadrimaculatus*), AeAChE (*Aedes aegypti*), AbAChE (*Aedes albopictus*).

<table>
<thead>
<tr>
<th></th>
<th>AgAChE</th>
<th>AaAChE</th>
<th>AqAChE</th>
<th>AeAChE</th>
<th>AbAChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propoxur</td>
<td>445 (267-623)</td>
<td>481 (319-643)</td>
<td>428 (335-521)</td>
<td>369 (304-433)</td>
<td>352 (257-447)</td>
</tr>
<tr>
<td>Carbofuran</td>
<td>47 (25-68)</td>
<td>55 (43-68)</td>
<td>43 (29-57)</td>
<td>41 (27-54)</td>
<td>46 (10-81)</td>
</tr>
<tr>
<td>Bendiocarb</td>
<td>172 (80-264)</td>
<td>182 (96-268)</td>
<td>137 (83-190)</td>
<td>127 (81-173)</td>
<td>137 (96-177)</td>
</tr>
<tr>
<td>PRC 331</td>
<td>104 (80-128)</td>
<td>100 (80-119)</td>
<td>93 (73-111)</td>
<td>79 (39-118)</td>
<td>87 (73-100)</td>
</tr>
<tr>
<td>PRC 337</td>
<td>476 (356-596)</td>
<td>379 (283-474)</td>
<td>390 (342-439)</td>
<td>275 (213-337)</td>
<td>332 (280-384)</td>
</tr>
<tr>
<td>PRC 388</td>
<td>221 (116-325)</td>
<td>217 (153-282)</td>
<td>281 (223-338)</td>
<td>341 (253-429)</td>
<td>288 (223-353)</td>
</tr>
<tr>
<td>PRC 408</td>
<td>106 (85-128)</td>
<td>93 (75-110)</td>
<td>92 (66-117)</td>
<td>208 (146-270)</td>
<td>177 (110-245)</td>
</tr>
<tr>
<td>PRC 421</td>
<td>431 (254-607)</td>
<td>546 (392-700)</td>
<td>565 (383-746)</td>
<td>756 (708-803)</td>
<td>574 (452-695)</td>
</tr>
</tbody>
</table>
Table 5-2. Mean (n=3) inhibition potencies of commercial and experimental methylcarbamates to agriculturally relevant insects.

<table>
<thead>
<tr>
<th></th>
<th><em>P. xylostella AChE</em></th>
<th><em>O. nubilalis AChE</em></th>
<th><em>D. citri AChE</em></th>
<th><em>A. mellifera AChE</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Propoxur</td>
<td>3322 (2524-4121)</td>
<td>6710 (4774-8646)</td>
<td>942 (689-1194)</td>
<td>941 (853-1030)</td>
</tr>
<tr>
<td>Bendiocarb</td>
<td>217 (143-291)</td>
<td>336 (229-442)</td>
<td>92 (62-121)</td>
<td>49 (34-64)</td>
</tr>
<tr>
<td>PRC 331</td>
<td>675 (370-980)</td>
<td>2933 (2541-3325)</td>
<td>128 (100-156)</td>
<td>32 (17-47)</td>
</tr>
<tr>
<td>PRC 337</td>
<td>1681 (1396-1965)</td>
<td>10847 (8148-13546)</td>
<td>1067 (812-1345)</td>
<td>379 (307-451)</td>
</tr>
<tr>
<td>PRC 388</td>
<td>3411 (3162-3660)</td>
<td>7794 (7073-8516)</td>
<td>210 (147-273)</td>
<td>77 (29-125)</td>
</tr>
<tr>
<td>PRC 408</td>
<td>52166 (42756-61578)</td>
<td>20640 (14831-26449)</td>
<td>3193 (2833-3552)</td>
<td>1242 (883-1601)</td>
</tr>
<tr>
<td>PRC 421</td>
<td>&gt; 100 uM</td>
<td>81940 (71788-92092)</td>
<td>6189 (5786-6591)</td>
<td>5440 (3990-6890)</td>
</tr>
</tbody>
</table>
Table 5-3. Mean (n=3) inhibition potencies of commercial and experimental methylcarbamates to AChE of model organisms. *TcAChE* inhibition values were previously reported in Jiang et al. (submitted in 2012).

<table>
<thead>
<tr>
<th></th>
<th><em>D. melanogaster</em> AChE</th>
<th><em>M. domestica</em> AChE</th>
<th><em>Human</em> AChE</th>
<th><em>T. californica</em> AChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propoxur</td>
<td>104 (48-159)</td>
<td>152 (106-199)</td>
<td>1442 (1255-1629)</td>
<td>1563 ± 145</td>
</tr>
<tr>
<td>Bendiocarb</td>
<td>27 (9-44)</td>
<td>58 (36-79)</td>
<td>182 (113-250)</td>
<td>126 ± 16</td>
</tr>
<tr>
<td>PRC 331</td>
<td>97 (66-129)</td>
<td>181 (142-220)</td>
<td>233 (154--311)</td>
<td>167 ± 27</td>
</tr>
<tr>
<td>PRC 337</td>
<td>131 (78-183)</td>
<td>156 (129-182)</td>
<td>8035 (7743-8327)</td>
<td>1300 ± 83</td>
</tr>
<tr>
<td>PRC 388</td>
<td>279 (315-343)</td>
<td>488 (434-541)</td>
<td>451 (431-470)</td>
<td>221 ± 40</td>
</tr>
<tr>
<td>PRC 408</td>
<td>23 (8-38)</td>
<td>51 (14-87)</td>
<td>5127 (4871-5383)</td>
<td>585 ± 83</td>
</tr>
<tr>
<td>PRC 421</td>
<td>482 (334-630)</td>
<td>654 (493-815)</td>
<td>112600 (72489-152711)</td>
<td>7430 ± 699</td>
</tr>
</tbody>
</table>
Table 5-4. Selectivity ratios (SR) obtained from *in vitro* inhibition potencies. SR values are expressed as follows: SR\(^1\) = *O. nubilalis* AChE IC\(_{50}\) / AgAChE IC\(_{50}\); SR\(^2\) = *D. citri* AChE IC\(_{50}\) / AgAChE IC\(_{50}\); SR\(^3\) = *A. mellifera* AChE IC\(_{50}\) / AgAChE IC\(_{50}\); SR\(^3\) = hAChE IC\(_{50}\) / AgAChE IC\(_{50}\)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>SR(^1)</th>
<th>SR(^2)</th>
<th>SR(^3)</th>
<th>SR(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propoxur</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Bendiocarb</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PRC 331</td>
<td>28</td>
<td>1.2</td>
<td>0.3</td>
<td>2</td>
</tr>
<tr>
<td>PRC 337</td>
<td>23</td>
<td>2.2</td>
<td>0.8</td>
<td>16</td>
</tr>
<tr>
<td>PRC 388</td>
<td>35</td>
<td>0.95</td>
<td>0.3</td>
<td>2</td>
</tr>
<tr>
<td>PRC 408</td>
<td>194</td>
<td>30</td>
<td>11</td>
<td>48</td>
</tr>
<tr>
<td>PRC 421</td>
<td>190</td>
<td>14</td>
<td>12</td>
<td>261</td>
</tr>
</tbody>
</table>
Table 5-5. Topical toxicity of methylcarbamates to three mosquito species and the European Corn Borer, *Ostrinia nubilalis*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th><em>An. gambiae</em></th>
<th><em>Ae. aegypti</em></th>
<th><em>Cu. Quinquefasciatus</em></th>
<th><em>O. nubilalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD$_{50}$ (ng/bug; 95% CI)</td>
<td>LD$_{50}$ (ng/bug; 95% CI)</td>
<td>LD$_{50}$ (ng/bug; 95% CI)</td>
<td>LD$_{50}$ (ng/bug; 95% CI)</td>
</tr>
<tr>
<td>Propoxur</td>
<td>3 (2-4)</td>
<td>5 (4-6)</td>
<td>20 (16-23)</td>
<td>1045 (931-1119)</td>
</tr>
<tr>
<td>Bendiocarb</td>
<td>2 (1-4)</td>
<td>2 (1-4)</td>
<td>6 (3-8)</td>
<td>101 (91-111)</td>
</tr>
<tr>
<td>PRC 331</td>
<td>4 (3-6)</td>
<td>7 (2-11)</td>
<td>14 (11-17)</td>
<td>2379 (2226-3134)</td>
</tr>
<tr>
<td>PRC 337</td>
<td>12 (8-17)</td>
<td>19 (10-25)</td>
<td>85 (74-96)</td>
<td>3310 (2905-3691)</td>
</tr>
<tr>
<td>PRC 388</td>
<td>8 (6-10)</td>
<td>12 (9-15)</td>
<td>92 (82-106)</td>
<td>3189 (2745-3609)</td>
</tr>
<tr>
<td>PRC 408</td>
<td>10 (5-17)</td>
<td>23 (19-28)</td>
<td>50 (45-56)</td>
<td>2842 (2503-3119)</td>
</tr>
<tr>
<td>PRC 421</td>
<td>81 (63-100)</td>
<td>92 (77-111)</td>
<td>83 (77-90)</td>
<td>3826 (3493-4580)</td>
</tr>
</tbody>
</table>
CHAPTER 6
CONCLUSIONS

The first objective of this dissertation research aimed to address potential interactions between solvent and target proteins during the high throughput screening process of insecticides. I performed enzyme kinetic studies to support the previous results of DMSO mediated antagonism of mosquito AChE inhibition (Swale, 2009). The bimolecular rate constant (ki) was utilized to determine the effect of 0.1 % DMSO on the carbamoylation reaction of AgAChE and supported the IC$_{50}$ data collected in Swale (2009). Both in vitro experimental systems showed a decreased inhibition of mosquito selective carbamates when in the presence of 0.1 % DMSO, but no inhibition differences were observed with non-selective carbamates or human AChE.

Molecular models suggest the selectivity of our novel carbamates and antagonist effects of inhibition is likely due to flexibility of W84 and W431 (Ag numbering) within the hydrophobic subpocket of AgAChE. The corresponding residues within hAChE are hydrogen bonded with Y449 (h), producing a rigid enzyme structure within the hydrophobic subpocket. Molecular models also suggest that DMSO is capable of hydrogen bonding with the indole nitrogens of W84/W431 (Ag numbering) and D441 within AgAChE, forming a DMSO stabilized ‘allosteric’ subpocket. This DMSO stabilization causes AgAChE to have a more rigid structure approaching that of hAChE, thereby inhibiting highly branched (selective) experimental carbamates from binding to the target site.

These findings have large implications for high-throughput screening processes of insecticides, usually performed by industrial chemical companies, as companies using one protocol to screen for selective mosquitocides, and specifically AChE, are likely discarding prospective lead compounds.
Future studies would be to continue to isolate the mechanism of antagonism toward inhibition through site directed mutagenesis. Substitution of Y449 of hAChE to an aspartate would allow for similar experiments to determine if the paralogous substitution is responsible for both, selectivity and antagonism of inhibition. Studies with bivalent inhibitors and/or tacrine dimers would also be useful to determine if the DMSO mediated stabalization would alter the enzyme structure to affect dual site binding of inhibitors.

The second objective of this dissertation research was two-fold. First, we characterized the inhibitor profile of acetylcholinesterases from *Boophilus microplus* (*BmAChE1*) and *Plebotomus papatasi* (*PpAChE*) compared to human and bovine AChE, in order to identify divergent pharmacology that might lead to selective inhibitors. Secondly, we performed a screen of highly potent and selective experimental carbamate inhibitors that can assist in the control of *Bm* and *Pp* populations.

Results indicate that *BmAChE1* has uniquely low sensitivity (IC$_{50}$ = 220 µM) toward tacrine, an inhibitor with nanomolar blocking potency in all previous species tested, including *Phlebotomus papatasi* (IC$_{50}$ = 205 nM). Molecular homology models indicate that the paralogous substitution W384/Y337 inhibits ligand binding in *BmAChE1*. In addition, *BmAChE1* and *PpAChE* display low nanomolar sensitivity to a variety of carbamate insecticides. **Compound 6**, 2-((2-ethylbutyl)thio)phenyl methylcarbamate (IC$_{50}$ = ca. 15 nM in both species), possesses greater than 350-fold selectivity for *BmAChE1* and *PpAChE* over mammalian AChE and a mouse oral LD$_{50}$ of greater than 1500 mg/kg.

These findings have significant implications for the future design of selective and resistance-mitigating inhibitors for the control of vectored diseases by *Bm* and *Pp*. The non-selective inhibitor, tacrine, yielded a 1000-fold difference in the inhibition profile for *BmAChE1*
over $Pp$AChE, indicating a unique AChE gorge geometry. The high potency of carbamates 6 toward $Bm$AChE1 and $Pp$AChE in conjunction with the low mammalian activity provides an attractive alternative and superior insecticide for $Bm$ and $Pp$ control.

Future work would be to isolate the remaining paralogous substitutions, other than W384, within the $rBm$AChE1 protein that reduces the binding activity of tacrine and bivalent inhibitors. Due to W384F only partially restoring the activity of tacrine, it is plausible to suggest other substitutions remain that could be utilized to gain a better understanding of the $Bm$AChE protein. A better understanding of the protein structure could then be used to create further selective and resistance mitigating inhibitors. After a clearer picture of the $Bm$AChE protein is determined, it would also be possible to design bivalent ligands that could take advantage of the unique paralogous substitutions within $Bm$AChE1. Also, due to the substantially increased selectivity and equipotency observed with carbamate 6 when compared to coumaphos oxon, it would be reasonable to analyze further analogs of ortho- substituted carbamates thiol linkers.

The third objective of this dissertation research was to characterize the toxicity and the mode of action of DEET neurotoxicity and compare its action to known anticholinesterases, local anesthetics, and octopaminergic chemical. Here the goal was to provide insights into the specific targets of DEET with respect to acute toxicity. Recent studies have also raised the possibility that DEET is an AChE inhibitor and that this action may contribute to its effects in insects, and cause risk of toxicity in exposed individuals. An understanding of DEET neurotoxicity is vital for its continued use as a repellent.

Results indicate that DEET is lethal to mosquitoes at topical doses in the microgram range (2-4 $\mu$g), but DEET is an extremely poor AChE inhibitor in mosquitoes ($<10\%$ inhibition), even at a concentration of 10 mM. AChE enzymes from human, $Drosophila$ melanogaster, and
Musca domestica are slightly more sensitive with IC$_{50}$ values ranging between 5 and 10 mM. Phentolamine was found to completely block the CNS neuroexcitation of DEET and octopamine, but was not found to be an effective blocker of propoxur. This finding suggests DEET is likely targeting octopaminergic synapses, and not acetylcholinesterase to induce toxicity.

To conclude, it is unlikely that DEET exerts its toxicity through anticholinesterase properties due to its low potency for enzyme inhibition, and the block of CNS neuroexcitation with phentolamine. Thus, it is plausible to suggest that toxicity to houseflies, and likely mosquitoes, is through a mimicking action of the neurotransmitter octopamine in the octopaminergic system as was portrayed with the CNS recordings performed with phentolamine. Numbness of mammalian mucous membranes is potentially explained through an anesthetic effect of nerve conduction block.

Future studies would be to determine the action of DEET within intact mosquitoes through in situ recordings of the flight motor reflex pathways using brain stimulation and recording dorsolongitudinal muscle activation. Such a study would provide evidence for the action of DEET is within the mosquito and could be compared to formamidine, carbamates, and anesthetic compounds. Also, to continue to isolate the potential DEET is acting on the octopaminergic system, studies could be performed using the firefly light organ as in this tissue the primary neurotransmitter is octopamine. Application of DEET would determine if it is capable of activating the lanterns. In vitro biochemical analyses could also be performed through binding studies of DEET to the octopamine receptor within the firefly lantern, and measuring cAMP production.
The final objective of this dissertation research was to determine the *in vitro* and *in vivo* activity of experimental insecticides to other nuisance biting mosquitoes, model organisms, and agricultural pests. These findings will provide us with a broader perspective on the potential toxicity of these compounds to other insects and could show advantageous properties for resistance management.

Accounting for mosquito resistance toward insecticides is vital when developing mosquitocides for disease control. Insecticide resistance of mosquitoes due to agricultural uses has been documented, and specifically effects insecticide design for disease control. Widespread agricultural use of pyrethroids has been implicated in exacerbating development of resistance to insecticides with the same mode of action when used in ITNs. Development of highly selective insecticides with poor toxicity to agricultural pests, and therefore less ancillary uses, can mitigate resistance through reduced selection pressure within breeding sites.

Results indicate unique insect selectivity among the novel carbamates, as they were found to be selective for mosquitoes over agricultural pests (190-fold) and human (260-fold) enzymes. Mouse oral toxicity showed promising results as the experimental inhibitors were at least 10-fold less toxic when compared to propoxur (24 mg/kg). The least toxic inhibitors to the mouse were found to be PRC 408 and PRC 421 with LD$_{50}$'s of 1700 mg/kg and >2000 mg/kg, respectively.

In conclusion, the potent AChE inhibition of mosquito AChE and previously undocumented levels of low mouse oral toxicity coupled with the low activity to agricultural insects suggest these are likely candidate insecticides to be employed into any mosquito control program. The general lack of activity against agricultural pests suggests avoidance of cross
resistance development in non-target species from incidental insecticide exposure from agricultural uses.

Future studies on the design of future mosquitocides would be to further analyze the ortho-substituted, branched carbamates as they comprise a promising approach to more selective carbamates against An. gambiae. Studies to determine the effectiveness and degree of resistance management through a combination of novel carbamates and pyrethroids would be of use to enhance the currently utilized control methods within Africa while retaining the low non-target toxicity shown in this study.
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BIOGRAPHICAL SKETCH

Daniel Swale was raised in a military household and lived in eight different states throughout his childhood and into his high school years. Today, he claims Virginia as his home and currently has family located in Yorktown, Virginia. Daniel graduated from Grafton High School in 2004 and attended Christopher Newport University where he majored in Biology and Chemistry with a pre-medical track. He participated in the NCAA varsity cross country and track teams all four years of his undergraduate education. Upon graduation, Daniel accepted a Master of Science graduate research assistantship at Virginia Tech in the laboratory of Professor Jeff Bloomquist to study insect toxicology and medical entomology. He graduated from Virginia Tech in December 2009 and followed Professor Bloomquist to the University of Florida. There he has continued his fascination with insects, biochemistry, organic chemistry, and toxicology through a pursuit of a Ph.D. in insect toxicology.

His career goals are to obtain a tenure-track professorship position in academia to study the control of vector borne diseases through the use of biochemical and toxicological methods.