Toxicological Analysis of Tacrines and Verapamils to the Yellow Fever Mosquito, *Aedes aegypti*

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ABSTRACT

Mosquitoes affect human health worldwide as a result of their ability to vector multiple diseases. Mosquitocide resistance is a serious public health challenge that warrants the development of improved chemical control strategies for mosquitoes. Previous studies demonstrate the mosquito blood-brain barrier (BBB) to interfere with the target-site delivery and action of anticholinesterase chemistries. The ATP-binding cassette (ABC) transporters are efflux proteins that assist in maintaining the BBB interface and serve as a first line of defense to mosquitocide exposures. To date, there are three subfamilies (ABC –B, -C, -G) of ABC transporters; however, knowledge of these chemistries interacting with mosquito ABC transporter(s) is limited. Here, I report that tacrine and *bis*(7)-tacrine are relative non-toxic anticholinesterases at solubility limits; however, the addition of verapamil enhances toxicity of both tacrine and *bis*(7)-tacrine to mosquitoes. Verapamil significantly increases the mortality of mosquitoes exposed to tacrine and *bis*(7)-tacrine compared to the tacrine- and *bis*(7)- tacrine-only treatments. Tacrine and *bis*(7)-tacrine reduce acetylcholinesterase activity in mosquito head preparations compared to the untreated mosquitoes; however, the addition of verapamil significantly increases the anticholinesterase activity of tacrine and *bis*(7)-tacrine compared to the tacrine-and *bis*(7)-tacrine-only treatments. Tacrine and *bis*(7)-tacrine increase ATPase activity in *Aedes aegypti* at lower concentrations compared to that of verapamil (Fig. 3). The

differential increase in ATPase activity suggests that tacrine and *bis*(7)-tacrine are more suitable substrates for ABC transporter(s) compared to verapamil and, thus, provides putative evidence that ABC transporter(s) is a pharmacological obstacle to the delivery of these anticholinesterases to their intended target site.

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GENERAL AUDIENCE ABSTRACT

Mosquitoes affect human health worldwide as a result of their ability to vector multiple diseases. Widespread resistance limits the use of current mosquitocide chemistries to reduce the risk of mosquito-vectored diseases. Thus, mosquitocide resistance is a serious public health challenge that warrants the development of improved mosquitocide control strategies for these disease vectors. Previous studies demonstrate the mosquito blood-brain barrier (BBB) to interfere with the target-site delivery and action of anticholinesterase chemistries. The ATP-binding cassette (ABC) transporters are efflux proteins that assist in maintaining the BBB interface of mosquitoes and serves as a first line of defense to mosquitocide exposures. To date, there are three subfamilies (ABC –B, -C, -G) of ABC transporters that are implicated to confer multi-specific mosquitocide resistance in mosquitoes; however, knowledge of these chemistries interacting with mosquito ABC transporter(s) is limited. My results suggest that ATP-binding cassette transporter positioned within the blood brain barrier could be involved in the BBB penetration issue of target site delivery of tacrines to the central nervous system. Additionally, I show that ABC transporters causes an increase in toxicity in tacrine based compounds in mosquitoes when exposed to a combination of a ABC transporter inhibitor such as verapamil. Overall, this research indicates that ABC transporters are potential pharmacological targets that may be used to enhance the effectiveness of insecticides. However, further research is needed to confirm

these targets before it could be used in commercial use. In a broader sense, this research further implicates ABC transporters as a possible resistance mechanism.

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CHAPTER 1

1.1 Introduction

The yellow fever mosquito, *Aedes aegypti*, affects millions of people worldwide. Dengue, chickungunya, and yellow fever are life-threatening diseases vectored by this mosquito species. There are few effective strategies to control malarial mosquitoes, including insecticide-treated nets (ITNs) and indoor residual spraying (IRS) (Okumu et al. 2011). However, there are numerous biochemical and physiological adaptations that allow *Aedes* mosquitoes to survive lethal exposures to current-use insecticides, including mechanisms of decreased insecticide response (i.e., target-site resistance) or mechanisms of decreased insecticide exposure (i.e., metabolic resistance) (Vontas et al. 2012).

The serine hydrolase acetylcholinesterase (AChE) of *Ae. aegypti* rapidly hydrolyzes the neurotransmitter acetylcholine (ACh), thereby terminating cholinergic nerve transmission. AChE is a proven target site for high efficacy insecticides (e.g., carbamate and organophosphate insecticides); however, widespread insecticide resistance limits their use for chemically based mosquito control strategies. For example, Wong et al. (2012) confirmed a single amino acid mutation of AChE (i.e., G119S) that confers target-site resistance in mosquito. In addition, these anticholinesterase insecticide-resistant mosquitoes are reported to have increased cytochrome P450 monooxygenase, esterase, and glutathione *S*-transferase metabolic activities compared to anticholinesterase insecticide-susceptible mosquitoes (Mutunga et al. 2015). Previously, a pharmacological approach with novel *bis*(n)-tacrine anticholinesterases was conducted to profile the AChE of mosquitoes (Anderson et al. 2009). The bivalent *bis*(n)-tacrines (n = 2 - 12

methylene linkers) showed a tether-length dependence towards AChE of multiple mosquito species, with greater maximal potency for *bis*(7)-tacrine compared to tacrine or other *bis*(n)-tacrine anticholinesterases (Anderson et al. 2009). While this information has implications for the identification of unique AChE target sites for the development of anticholinesterase insecticides for mosquitoes, neither tacrine nor *bis*(7)-tacrine was lethal to the mosquitoes. Mutunga et al. (*2013*) report that the lack of observed mosquito toxicity to the *bis*(n)-tacrines might be due to the inability of the chemical to penetrate the blood-brain barrier and, in turn, impedes the target-site delivery of these anticholinesterases.

The blood-brain barrier of insects is composed of ATP-binding cassette (ABC) transporters that prevent the uptake and elimination of insecticides, thereby inhibiting the ability to target the central nervous system (Porretta et al. 2008). These ABC transporters serve as the first line of cellular defense for insects exposed to insecticides. ABC transporters that confer a multi-drug resistance (MDR) phenotype are of particular interest and have often been associated with insecticide resistance (Buss et al. 2002, Poretta et al. 2008). In *Ae. aegypti*, there are currently 44 ABC transporter proteins described within at least three subfamilies that confer a multi-drug resistance phenotype, including (i) 5 ABC sub-family B transporters for the efflux of hydrophobic insecticides, (ii) 14 ABC sub-family C family transporters for the export of insecticide conjugates (e.g., glutathione conjugates), and (iii) 12 ABC sub-family G family transporters with broad substrate specificity for the efflux of insecticides (Roth et al. 2003). To date, ABC transporter-mediated insecticide resistance has been reported for a small number of insect pests (Buss et al. 2002, Porretta et al. 2008); however, the interaction of ABC transporters towards tacrine-based compounds that inhibit mosquito AChE remains unknown.

The overall hypothesis behind this proposed research is that ABC transporters impede the ability of tacrine-based compounds to inhibit AChE and, thus, reduce the toxicity of these anticholinesterases to *Ae. aegypti*. Based on the above observations, the experimental focus was to examine the possible link between ABC transporter activity and AChE inhibition of these mosquitoes. The objectives of this research project were to examine (i) the individual acute toxicity, (ii) the binary acute toxicity, (iii) the AChE inhibition and (iv) the ATPase activity of *Ae. aegypti* exposed to verapamil (ABC transporter inhibitor) and tacrines (AChE inhibitors).

CHAPTER 2

LITERATURE REVIEW

2.1 Expansion of Mosquito Vectored-Diseases

The geographic distribution of numerous arthropod vectors and diseases has expanded worldwide causing a dramatic increase in cases and epidemics (Gubler et al. 2002). Previous public health policy and eradication control programs have reduced cases of vector-borne diseases in certain areas. Chemical control programs started in the 1900s after mosquitoes were discovered to transmit malaria parasites, making eradication an important concentration. However, the reductions of prevention efforts, increase of insecticide resistance, and change in human expansion have caused a resurgence or emergence of diseases. This change has allowed mosquitoes to overcome geographic barriers and adapt physiologically to diverse environmental conditions (Tatem et al. 2006), leading to emergence of vector-borne diseases. Recent biological invasions have included both insect disease vectors and the expansion or reemergence of vector-borne diseases. The change in human global air travel and application of chemical control programs reduced populations of insect-vectored diseases. Direct exposure to humans or animals infected with diseases increased the virus reproductive numbers and transmission rate from insects (Gubler et al. 2002). Due to their host preference variability, variation in mosquito behavior, vector biodiversity, and ecological adaptations, mosquitoes are the most important vector-borne arthropods that affect humans (Ferguson et al. 2010). The main impact on vector-borne disease viability comes from insecticide resistance resulting in an increase of mosquito populations. Mosquitoes affect millions of people worldwide as a result of their ability to vector multiple infectious diseases such as dengue, yellow fever, and chickungunya (Hemingway et al. 2006). Dengue is consider the most important arthropod borne disease in the 20th century (CDC

2010). With no effective treatment or commercial vaccine available, control strategies are focused on its primary mosquito vector, *Aedes aegypti* (WHO 2009, Bhatt et al. 2013).

2.2 Mosquito Borne Diseases

Yellow fever, dengue, chikungunya, and zika fever are life-threating diseases vectored by the *Aedes* mosquitoes, principally *Aedes aegypti*. This mosquito is widely distributed around the world with numbers increasing annually. Dengue is estimated to infect 390 million people yearly or 40% of the world's populations are at risk for endemic transmission (Bhatt et al 2013). There is currently no effective vaccine or drug treatment, therefore, prevention is focused on supportive care of symptoms to treat dengue infections (WHO 2009). Dengue, an arbovirus in the family Flaviviridae is transmitted by *Ae. aegypti*. There are four serotypes of the dengue virus, Den 1,2,3,4. *Ae. aegypti* mosquitoes thrive in both urban and rural area. Current control methods are focused on chemical, biological, and vector control of the *Aedes* mosquitoes. However, a surge of transmission rates and an increase in outbreaks of infection numbers have resulted from misuse of insecticides, financial burdens, and low prevention methods in developing and underdeveloped countries.

2.3 Insecticide Resistance

2.3.1 Chemical Control Strategies

The mosquito central nervous system is a proven target site for high efficacy insecticides, including carbamate-, organophosphate-, and pyrethroid-class chemistries. These insecticides work to kill mosquitoes by targeting acetylcholinesterase (AChE) and voltage-gated sodium channels (VGSC). Carbamate and organophosphate insecticides are AChE inhibitors that cause

acetylcholine to accumulate at the nerve synapse resulting in excessive excitation of cholinergic receptors leading to paralysis and death of the mosquito (Casida et al. 2004). Pyrethroids target the VGSC to extend the course of sodium current depolarization of the axon, thereby resulting in uncontrolled and persistent excitation of the neurons (Bloomquist 1999). There are few effective strategies to control *Ae. aegypti*, including insecticide-treated nets (ITNs) and indoor residual spraying (IRS) (Okumu et al. 2011). There are numerous biochemical and physiological adaptations that allow mosquitoes to survive lethal doses of current-use insecticides, including mechanisms of decreased insecticide response (i.e., target-site resistance) or mechanisms of decreased insecticide exposure (i.e., metabolic resistance) (Vontas et al. 2012).

2.3.2 Metabolic Detoxification

The biochemistry of insecticide resistance is important to understand the detoxifying enzymes when attempting to mitigate resistance through the manipulation of components or compound chemistries. Glutathione S-transferases (GST), esterases, and cytochrome P450 monooxygenases are three major enzyme groups responsible for metabolic resistance to organophosphates, organochlorines, carbamates, and pyrethroids (Hemingway et al. 2002). These enzymes recognize and modify compounds to reduce biological activity and decrease lipophilicity (Phase I) and conjugate metabolites to endogenous molecules for excretion (Phase II) (Casida et al. 2004). The effectiveness of these insecticides has diminished as a result of the increasing incidence of either target-site or metabolic detoxification resistance.

Biochemical characterization of metabolic resistance arising from cytochrome P450 monooxygenases, carboxylesterases, and glutathione *S*-transferases are groups of enzymes found

in the majority of animals, including insects to be responsible for metabolic resistance (Hemingway and Ranson, 2000). The cytochrome P450 monooxygenases are dimeric multi-functional enzymes that are involved in the detoxification of a wide range of insecticides (Prapanthadara et al. 1996). These enzymes can increase resistance by activating or deactivating the main toxic metabolite of the insecticide into a more hydrophilic and normally less toxic substrate that does not react the target site (Hemingway 2000). Resistance to insecticides are associated with pyrethroid resistance due to over expression of cytochrome P450 monooxygenases leading to increased metabolic breakdown of insecticides for detoxification purposes (Scott et al. 2015). The cytochrome P450 monooxygenases have been implicated in the development of insecticide resistance in *Aedes* mosquitoes (Ranson 2000). Cytochrome P450 monooxygenases are a group of enzymes found in most organisms, including insects. These enzymes play a role in the metabolism of insecticides and are involved in endogenous metabolism of compounds. The catalytic action of cytochrome P450 monooxygenases enzymes is through the oxygenation of an insecticide via uptake of electrons from NADPH (Hemingway et al. 2000). Insecticide resistance has been associated with the activation of cytochrome P450 monooxygenasess leading to increased expressions of ABC transporter proteins to excrete addition insecticides (Bariami et al. 2012; Dauchy et al. 2008).

Likewise, the glutathione *S*-transferases (GST) are dimeric multi-functional enzymes that are involved in the detoxification of a wide range of insecticides (Prapanthara et al., 1996; Ranson et al. 2001). These enzymes can increase resistance by conjugating glutathione (GSH) with the alkyl group of the insecticide or with the leaving group of the main toxic metabolite (Hemingway 2000). GSTs play a role in insecticide resistance due to elevated levels of

metabolic activity increase reductive dehydrochlorination using reduced glutathione, to produce water soluble metabolites that are readily excreted in many insect species, thereby preventing the insecticide to reach the target site (Yu et al. 1996; Enayati et al. 2005).

A wide range of medically important insect species, including mosquitoes, have been found to exhibit general esterase-based resistance to multiple insecticide groups such as organophosphate, carbamate, and pyrethroid insecticides (Hemingway et al. 2006, Hemingway et al. 2002). General esterases are a group of enzymes that work by hydrolyzing carboxylic esters in insecticides (Hemingway et al. 2000). Resistances to insecticides have been associated with esterases due to its ability to rapid sequestration rather than metabolization of the insecticide. The increase amplification of general esterase genes has been found in bed bugs, midges, and mosquitoes with enhanced hydrolysis and sequestration of organophosphates, carbamates, and pyrethroids (Adelman et al. 2011; McCarroll et al. 2000; Jin-Clark et al. 2008; Yu et al 2008).

Insecticide resistance due to reduced cuticular penetration is a common mechanism of resistance in insect populations. Reduced cuticular penetration provides slight resistance to insecticide exposures; however, in combination with other insecticide resistance mechanisms, it confers considerable resistance to insecticide exposures (Yu 2008; Wood et al. 2010). The reduced cuticular penetration of insecticides is suggested to be due to the affinity of the binding protein, lipid composition to prevent the penetration of insecticide, a degrading enzyme, or an impermeable layer of the cuticle (Yu et al. 2008). Structure activity relationships have suggested that the more polar molecules are more capable of penetrating the cuticle but are quickly dissociated from the intracellular environment, reducing toxicity (Dauchy et al. 2008; Vision and

Law 1971). These mechanisms have been implicated for insecticide resistance intobacco budworm, mosquitoes, bollworm, cabbage lopper and several other insect species (Anderson et al. 2009; Vinson and Law 1971; Ahmad et al. 2006; Tak et al. 2015).

Acetylcholinesterase (AChE) is a proven target site for high efficacy insecticides, including carbamates and organophosphates; however, widespread insecticide resistance limits their use for chemically based mosquito control strategies. For example, Wong et al. (2012) recently confirmed a single amino acid mutation of AChE (i.e., G119S) that confers target-site resistance in mosquitoes. Previously, a pharmacological approach with novel *bis*(n)-tacrine anticholinesterases was conducted to profile the AChE of mosquitoes (Anderson et al. 2009). The bivalent *bis*(n)-tacrines (n =2 – 12 methylene linkers) showed a tether length dependence towards mosquito AChE, and other mosquito species, with greater maximal potency for *bis*(7)-tacrine compared to tacrine or other *bis*(n)-tacrine anticholinesterases (Anderson et al. 2009). While this information has implications for the identification of unique AChE target sites for the development of AChE-inhibiting insecticides for mosquitoes, neither tacrine nor *bis*(7)-tacrine was lethal to the mosquitoes. Mutunga et al. (2013) findings suggest the blood-brain barrier can prevent the penetration of the *bis*(n)-tacrines, thereby, impeding the target-site delivery of these anticholinesterases to the central nervous system, consistent with the lack of observed toxicity.

2.3.3 Mosquito ABC Transporters

The blood-brain barrier of insects is composed of ATP-binding cassette (ABC) transporters that prevent the uptake and elimination of insecticides, thereby inhibiting their ability to target the central nervous system (Porretta et al. 2008). The ABC transporters may serve as the first line of

cellular defense for insects exposed to insecticides. The ABC transporters that confer the multi-drug resistance (MDR) phenotype, or P-glycoproteins (P-gps), are of particular interest and have often been associated with insecticide resistance (Buss et al. 2002, Poretta et al. 2008). ABC transporters have been typically associated in the gut, malpighian tubules, and the cuticular epithelium of insects (Broehan et al 2013; Labbe et al 2010; Mayer et al. 2009; Leader and O'Donnell 2005). To date, ABC transporter(s)-mediated insecticide resistance has been reported for a small number of medically important insect pests (Buss et al. 2002, Porretta et al. 2008). Recent reports have revealed ABC transporter(s) modification of insecticide toxicity in *Ae. caspius* and *Culex pipens* (Buss et al. 2002, Porretta et al. 2008). Insecticide resistance has been shown in species with an over-expression of ABC transporters (Simmons et al. 2013; Figueira et al 2013). ABC transporters function by hydrolyzing ATP and moving chemicals and other substrates across cellular membranes (Lopez et al 2014). For example, Figueria et al. (2013) reported that insecticides are able to increase the toxicity of tempos by 24% when in combination with the ABC transporter inhibitor verapamil. However, the interaction of ABC transporters towards anticholinesterase insecticides for mosquito control remains unclear.

CHAPTER 3

INCREASED TOXICITY OF TACRINE AND *BIS*(7)-TACRINE IN COMBINATION WITH VERAPAMIL TOADULT MOSQUITOES

3.1 Introduction

The *Aedes* mosquitoes affect millions of people worldwide as a result of their ability to transmit dengue fever, chikungunya, and yellow fever, thus posing a major long term public health problem for numerous countries (Vontas et al. 2012). Dengue fever, yellow fever, and dengue-dengue hemorrhagic fever are considered the most important mosquito-borne vectored diseases that affect humans (CDC 2014). Dengue affects nearly one million people annually and an estimated 3 billion people or 40% of the world's population, are at risk for endemic transmission (Bhatt et al 2013). The mosquito nervous system is a proven target site for high efficacy chemistries, including the carbamate-, organophosphates-, and pyrethroid-class insecticides (Anderson et al. 2009, Hartsel et al. 2012, Wong et al. 2012). However, both target-site and metabolic resistance limits the use of these insecticidal chemistries to reduce the risk of *Aedes*-borne diseases. Thus, new and improved chemical control strategies are required for vector mosquitoes.

Acetylcholinesterase is a target site for carbamates and organophosphate insecticides; however, widespread insecticide resistance limits their use for chemically based mosquito control strategies. For example, Wong et al. (2012) recently confirmed a single amino acid mutation of AChE (i.e., G119S) that confers target site resistance in mosquitoes. Previously, a

pharmacological approach with novel *bis*(n)-tacrine anticholinesterases was conducted to profile the AChE of mosquitoes (Anderson et al. 2009). The bivalent *bis*(n)-tacrines (n = 2 – 12 methylene linkers) showed a tether length dependence towards mosquito AChE, and other mosquito species, with greater maximal potency for *bis*(7)-tacrine compared to tacrine or other *bis*(n)-tacrine anticholinesterases (Anderson et al. 2009). While this information has implications for identification of unique AChE target sites for the development of anticholinesterase insecticides for mosquitoes, neither tacrine nor *bis*(7)-tacrine was found to be lethal to the mosquitoes. Mutunga et al. (2013) report that the lack of observed mosquito toxicity to the tacrines is due to their inability to penetrate the blood-brain barrier of insects, thereby impeding the target site action of these anticholinesterases.

The blood-brain barrier of insects is composed of ATP binding cassette (ABC) transporters that prevent the uptake and elimination of insecticides, thereby inhibiting their ability to target the central nervous system (Porretta et al. 2008). These ABC proteins are present in all kingdoms of life and are one of the largest transporter families (Dermauw and Van Leeuwen 2013). The majority of these ABC proteins hydrolyze ATP to transport substrates or non-substrates across lipid membranes as primary active transporters (Demauw and Van Leeuwen 2013). Thus, ABC proteins may serve as the first line of cellular defense for insects exposed to insecticides. The ABC transporters that confer the multi-drug resistance (MDR) phenotype, also known as P-glycoprotein (P-gp), are of particular interest and have often been associated with insecticide resistance (Buss et al. 2002, Poretta et al. 2008). To date, ABC transporters-mediated insecticide resistance has been reported for numerous medically important insect pests (Buss et al. 2002, Porretta et al. 2008). Recent reports have revealed ABC transporters modification of insecticide

toxicity in *Aedes caspius*, *Culex pipens*, and *Ae. aegypti* (Buss et al. 2002, Porretta et al. 2008, Figueria-Mansur et al. 2013). The increased expression of ABC transporer(s) has been reported in insecticide-resistant insects (Simmons et al. 2012). In addition, ABC transporters have been shown to have multiple primary active drug-binding transport sites on the transmembrane domain (Labbe et al. 2013; Zhang et al 2003; Dermauw and Van Leeuwen 2013). Photonaffinity labeling studies have revealed up to seven different drug binding sites associated with ABC transporters which contain multiple binding sites for different substrates and non-substrates (Zhang et al. 2003; Safa 2004). Substrates and modulators can bind to the secondary binding sites that cooperate with drug binding or transport sites (Lespine et al. 2012). However, the interaction of mosquito ABC transporters with anticholinesterase compounds remains unclear.

This study examined the acute toxicity of tacrine and *bis*(7)-tacrine (AChE inhibitors) alone and in combination with verapamil (ABC transporter inhibitor) to the yellow fever mosquito, *Ae. aegypti*. Herein, I report the: 1) individual acute toxicity of tacrine, *bis*(7)-tacrine, and verapamil to *Ae. aegypti*, and 2) the binary acute toxicity of tacrine, *bis*(7)-tacrine, and verapamil to *Ae. aegypti*.

3.2 Material and Methods

3.2.1 Chemicals

Bis(7)-tacrine, dimethyl sulfoxide (DMSO), ethanol, magnesium chloride ($MgCl_2$), and tacrine were purchased from Fisher Scientific (Pittsburg, PA). Acetylthiocholine iodide (ATCh), adenosine 5'triphosphate disodium salt (ATP-Na), 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), ethylene glycol-*bis*(2-aminoethylether)-N,N,N'N'-tetraacetic acid(EGTA), *n*-phenylthiourea,

protease inhibitor cocktail, potassium nitrate (KNO_3), phenylmethylsulfonyl fluoride (PMSF), sodium azide, sodium dodecyl sulphate (SDS), thapsigargin, trizma base (Tris hydrochloride, Tris-HCl), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Ouabain was purchased from Chem Service (West Chester, PA). Verapamil was purchased from INDOFINE Chemical Company, Inc. (Hillsborough, NJ).

3.2.2 Mosquito Colony Maintenance

A laboratory colony of *Aedes aegypti* (Liverpool strain) initially obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (Manassas, VA), has been cultured in the Insect Toxicology and Pharmacology Laboratory at Virginia Tech (Blacksburg, VA) since 2011. The mosquito colony was maintained according to the standard operating procedures described by Pridgeon et al. (2009) with slight modifications. The adult mosquitoes were held in a screened cage and provided 10% sucrose *ad libitum*. To encourage egg development in adult females, a membrane feeder containing defribrinated sheep blood (Colorado Serum Co., Denver, CO) was warmed to 37 °C and provided to the mosquitoes. The mosquito eggs were collected from the adult females and vacuum-hatched in a 1-L Erlenmeyer flask. The mosquito larvae were transferred to a plastic tray containing deionized water. A larval diet of flake fish food (Tetramin, Blacksburg, VA) was added to the plastic tray. The mosquito adults and larvae were reared in an environmental chamber at 28 °C and 75% relative humidity with a 16 h:8 h (light:dark) photoperiod.

3.2.3 Individual Acute Toxicity Bioassays

The acute toxicity bioassays were conducted for 24 h using 3-5 d old adult female mosquitoes exposed to six doses of tacrine, *bis*-7-tacrine, and verapamil to provide a range of 0-100% mortality. The appropriate dilutions of each compound were prepared in ethanol. Non-blood fed mosquitoes were collected with an aspirator and anesthetized on 4 °C chill plates. The compounds were delivered by adding 0.2 µl of compound solution to the dorsal thorax of 10 mosquitoes per dose using a Hamilton PB600 repeating dispenser and a 700 series syringe (Reno, NV). The same procedure was used to treat mosquitoes with a corresponding dose of ethanol as a control treatment. The bioassays were repeated three times for each compound dose and control treatment. Treated adults were provided 10% sucrose solution and maintained in plastic cups in an environmental chamber at 28 °C and 75% relative humidity with a 16 h:8 h (light:dark) photoperiod. The endpoint for each bioassay was measured as a lethal dose (LD) according to World Health Organization guidelines (WHO 2006). Adult mosquito that was unable to right themselves were considered dead.

3.2.4 Binary Toxicity Bioassays

The binary effect of tacrine, *bis(7)*-tacrine and verapamil was examined in 3-5 d old adult female mosquitoes treated with tacrine or *bis*(7)-tacrine at the solubility limit individually, 10 µg/mosquito or 5 µg/mosquito, respectively and in combination with four treatments of verapamil. The different doses of verapamil were applied in 0.2 µl aliquots to the dorsal thorax of 10 mosquitoes per dose one hour prior to treatment with the solubility of tacrine or *bis*(7)-tacrine using a Hamilton PB600 repeating dispenser and a 700 series syringe (Reno, NV). The same procedure was used to treat mosquitoes with ethanol as a control treatment. The magnitude of altered toxicity was examined in female adult mosquitoes exposed to six doses, respectively, of tacrine and *bis*-7-tacrine individually and in combination with a single dose of verapamil.

Twenty-five mosquitoes were treated per replicate and repeated three times. Control treatments included an untreated, solvent (ethanol), and a verapamil control at 20 µg. The adult toxicity bioassays were conducted using the same procedures as those described above.

3.2.5 Statistical Analysis

A log-probit analysis using PoloPlus was used to estimate the LD_{50} values for tacrine, *bis*(7)-tacrine, and verapamil for the individual and binary acute toxicity bioassays. The significant differences between the LD_{50} for tacrine, *bis*(7)-tacrine, and verapamil individually and in combination with verapamil were based on the non-overlapping 95% confidence intervals estimated for each bioassay. The percentage of mosquitoes affected by the differences between each treatment were statistically compared to the control treatment using a one way analysis of variance (ANOVA) in combination with a Tukey's multiple comparison test.

3.3 Results

3.3.1 Individual Acute Toxicity Bioassays

Tacrine, *bis*(7)-tacrine, and verapamil were not acutely toxic, up to solubility limits in ethanol, to the mosquitoes under the tested bioassay conditions (Table 1). However, the acute toxicity of tacrine and *bis*(7)-tacrine was increased when applied in combination with verapamil, with 24-hr LD_{50} values of 6.31 and 1.86 µg/mosquito, respectively (Table 1). Tacrine at 10 µg/mosquito in combination with verapamil at 0.1, 1, and 10 µg/mosquito significantly increased mosquito mortality 2.8-, 4.0-, and 5.2-fold, respectively, compared the tacrine-only treatment (Fig. 1). *Bis*(7)-tacrine at 5 µg/mosquito in combination with verapamil at 1 and 10 µg/mosquito

significantly increased mosquito mortality by 1.8- and 3.8-fold, respectively, compared to the *bis*(7)-tacrine-only treatement (Fig. 1).

3.4 Discussion

Due to the wide extent of insecticide application worldwide, insecticide resistance has resulted in significant emergence and re-emergence of diseases. Current chemical control strategies are concentrated on insecticide resistance such as target site and metabolic resistance. Previous studies have determined insecticide penetration issues due to the blood brain barrier (Mutunga et. al 2013). The blood-brain barrier of insects is composed of ATP binding cassette (ABC) transporters that prevent the uptake and elimination of insecticides, thereby inhibiting their ability to target the central nervous system (Porretta et al. 2008). This study aimed to provide evidence of ABC transporter modulation of insecticide transport in *Aedes aegypti* mosquitoes. We used tacrine and *bis*(7)-tacrine as our target site compound due to previous studies using these anticholinesterases to pharmacologically map the acetylcholinesterase active site gorge (Wong et al 2012). Verapamil was used to first determine the role of ABC transporter inhibitor in modulation of these insecticides in *Ae. aegypti*. This finding may provide further insights into ABC transporter interactions in mosquitoes and into drug resistance.

Here, I report that tacrine and *bis*(7)-tacrine up to 10 µg/mosquito and 5 µg/mosquito, respectively, did not show acute toxicity to mosquitoes, *Ae. aegpyti*. However, we have determined that the toxicity of tacrine and bis(7)-tacrine was increased by 2- and 22-fold when in combination with verapamil (20 µg) as compared to anticholinesterase inhibitor–only treatments (Table 1). This suggest that the addition of the ABC transporter inhibitor verapamil enhances the

toxicity of tacrine and *bis*(7)-tacrine to *Ae. aegypti.* We determined that verapamil significantly increases the mortality of *Ae. aegypti* exposed to tacrine, when in combination with verapamil, by as much as *ca*. 77% compared to the tacrine-only treatment (Fig. 1). The mortality of mosquitoes exposed to *bis*(7)-tacrine in combination with verapamil was increased by *ca*. 60% compared to the *bis*(7)-tacrine-only treatment (Fig. 1).

This study indicates that the altered toxicity observed between verapamil and these anticholinesterases are associated with ABC transporter interference with transport of insecticides to their intended target site. This indicates that poor blood brain barrier penetration could be due to a possible resistance mechanism by mosquito ABC transporter to transport out substrates and non-substrates.

This study showed that tacrine and *bis*(7)-tacrine increase ATPase activity in *Ae*. *aegypti* at lower concentrations, compared to that of verapamil (Fig. 3). A previous study has demonstrated that suitable substrates such as verapamil are capable of facilitating reduced ATPase activity across the ABC transporters through blocking of active ATP sites (Taub et. al 2005). The differential increase in ATPase activity suggests that tacrine and *bis*(7)-tacrine are more suitable substrates for ABC transporters compared to verapamil and thus provide putative evidence that ABC tranporters are a pharmacological obstacle to the delivery of these anticholinesterases to their intended target site. These data are in alignment with our previous studies that have confirmed the blood-brain barrier of *Drosophila melanogaster* to be a limiting factor in tacrine anticholinesterase toxicity (Mutunga et al. 2012).

In summary, this study reveals that verapamil significantly modifies the toxicity of anticholinesterase and that this altered toxicity may be the result of ABC transporters affecting the transport of compounds across the blood brain barrier. This study provides putative evidence for insecticide transport substrates (tacrine and *bis*(7)-tacrine) and inhibitory ligands (verapamil) of *Ae. aegypti* ABC transporter and thus demonstrates a possible role for these proteins to impede the transport of the anticholinesterases to their target site (AChE) within the mosquito central nervous system. Current research activities are focused on the molecular characterization of mosquito ABC tranporter(s) as a first step towards the identification of ABC transporter interactions and the validation of potential resistance mechanisms against insecticidal chemistries. Further analysis should be conducted to determine the relationship between selective ABC transporter expression and their potential for drug transport. The long term goal of this research study is to provide a better understanding of "chemomodulators" that might be used to enhance the effectiveness of insecticides that are ABC transporter substrates (e.g., pyrethroids and carbamates for mosquito control).

196 **Table 1.** Toxicity of tacrine, *bis*(7)-tacrine, and verapamil, alone and in combination, to adult mosquitoes, *Ae. aegypti*.

Compound[a]	No.	LD_{50} (µg/mosquito)[b]	95% CI	Slope ± SE
Tacrine	240	30.00 ± 7.07% Mortality	N/A	N/A
Tacrine + Verapamil	240	6.31	5.83 - 6.81	7.54 ± 1.36
Bis(7)-Tacrine	240	27.50 ± 4.79% Mortality	N/A	N/A
Bis(7)-Tacrine + Verapamil	240	1.86	1.45 - 2.26	3.18 ± 0.52
Verapamil	240	10.00 ± 4.08% Mortality	N/A	N/A

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198 [a]Each compound was topically administered as 0.2-µl aliquots to the dorsal thorax of the mosquitoes for 24 h.

199 [b]Tacrine, *bis*(7)-tacrine, and verapamil individual and binary acute toxicity data are presented as LD_{50} and their 95% confidence
200 intervals (95% CI) in micrograms per mosquito (µg/mosquito), the dose at which 50% of the tested larvae were dead, respectively,
201 in a 24-h bioassay. Tacrine, *bis*(7)-tacrine, and verapamil were tested at solubility limits in ethanol and reported as percent
202 mortality ± standard error (n = 3). Log-probit analysis was used to estimate the endpoint dose for each compound.

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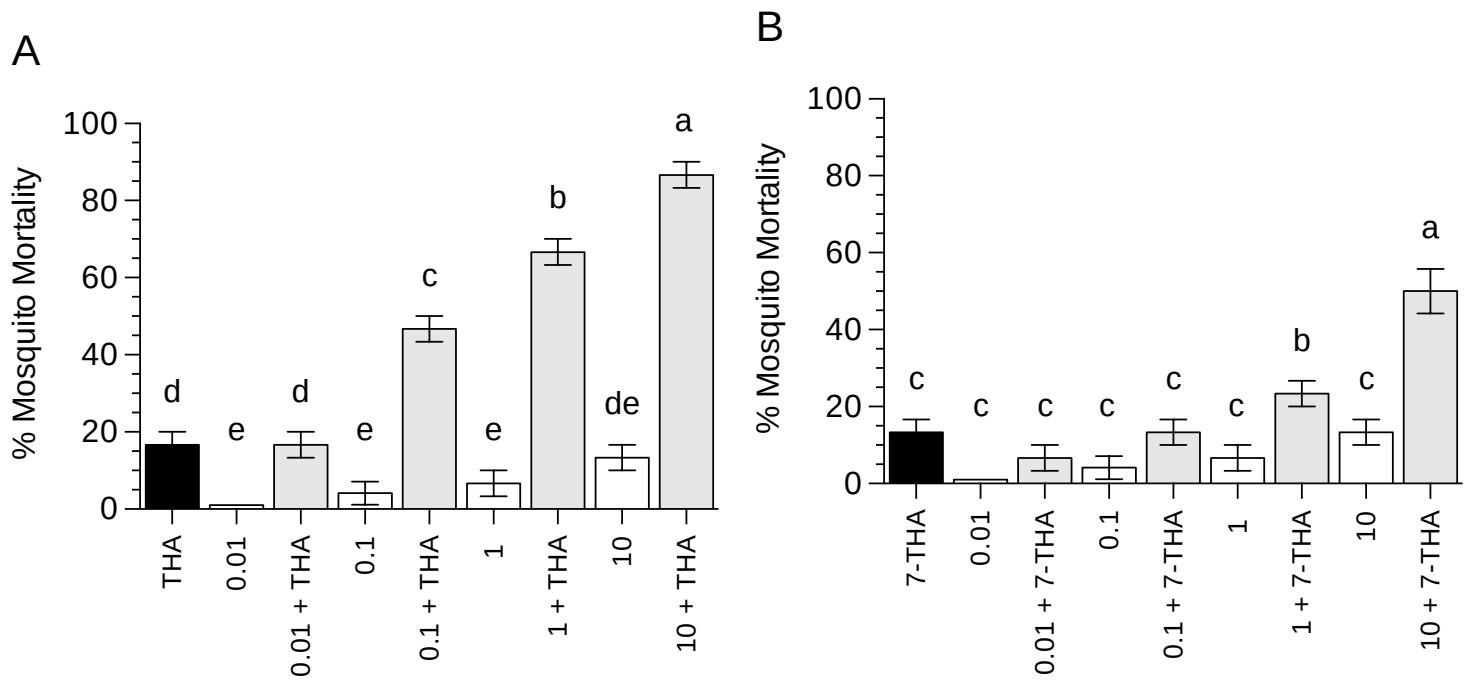

Figure 1. Percent mortality of adult mosquitoes, *Ae. aegypti*, exposed to A) tacrine (THA) at 10 µg/mosquito or B) *bis*(7)-tacrine (7-THA) at 5 µg/mosquito alone and in combination with verapamil (VER) at 0.01, 0.1, 1, and 10 µg/mosquito. Vertical bars indicate standard errors of the mean (n = 4). Different letters on the bars indicate that the means are significantly different among the treatments using a one-way analysis of variance and Tukey's multiple comparison test ($p < 0.05$).

CHAPTER 4

ACETYLCHOLINESTERASE AND ABC TRANSPORTER ATPASE ACTIVITY IN MOSQUITOES TREATED WITH TACRINE, *BIS*(7)-TACRINE, AND VERAPAMIL

4.1 Introduction

Insecticide resistance is increasing at significant rate due to re-emergence of mosquito-borne diseases such as dengue, zika virus, and chikungunya, which are transmitted by the *Aedes aegypti* mosquito (Luz *et al*., 2011; Vontas *et al*., 2012). The extensive use of insecticides coupled with genetic modifications has decreased susceptible *Ae. aegypti* populations to several insecticides, thereby limiting their use. The lack of effective vaccine and treatments results in a demand for an alternative method to reduce disease transmission. These mosquito species are domesticated and have evolved to breed in assorted environments such as used car tires, old plastic cartons, tree holes, flower vases, and small crevices (Tatem et al. 2006; Ferguson et al. 2010). Resistance to insecticides can be caused by different mechanisms including reduced insecticide penetration through the cuticle, modification of the insecticide target site, and metabolic resistance through detoxification enzymes such as cytochrome P450s, general esterases and glutathione *S*-transferases (Hemingway and Craig, 2004; Ranson and Hemingway, 2005; Hartsel et al 2012; Mutunga et al. 2015). Due to these factors, the understanding of insecticide resistance mechanism is essential for the development of chemical control strategies and development of novel insecticides for the control of disease vectors worldwide.

Acetylcholinesterase is a highly effective target-site for two major insecticides: carbamates and organophosphates. AChE is critical for sustaining life within mammals and insects due to the regulation of neurotransmitter acetylcholine (ACh) (Weill et al 2002; Mutunga et al. 2015). It serves to hydrolyze ACh within cholinergic synapses by terminating synaptic transmission in the central nervous system (Anderson et al. 2009; Mutunga et al. 2013; Wong et al. 2009). Inhibition of AChE will lead to repeated nerve stimulation, paralysis, and eventual death of the insect. Although many anticholinesterases inhibitors have high insect toxicities, target-site insensitivity (G119s mutation) has reduced the efficacy of the AChE targeted insecticides (Wong et al. 2009). The emergence of carbamate- and organophosphate-resistant mosquito populations has led to the characterization of AChE target site.

A pharmacological approach with experimental compounds using *bis*(n)-tacrines was used to map the catalytic gorge of acetylcholinesterase in humans and several species of mosquitoes (Anderson et al. 2009). These *bis*(n)-tacrines are two tacrine dimers connected with 2-12 methylene linkers. A greater maximal potency for *bis*(7)-tacrine was observed in mosquitoes, indicating a tether length dependency (Anderson et al. 2009). However, due to their lack of toxicity to live mosquitoes, Mutunga et al. (2013) suggests the lack of toxicity is likely due to the blood-brain barrier preventing penetration of dimeric tacrines into the CNS. Results discussed in Mutunga et al. (2013) reported greater firing frequency in the transected *Drosophila melanogaster* CNS compared to the intact CNS, consistent with the lack of observed toxicity.

In all domain of life (bacteria, archae, eukarya), multidrug resistance (MDR) is often related to the ATP-binding cassette (ABC) transporter protein family, the largest transporter family

(Demauw et al 2013). The ability of ABC transporters to effectively limit the penetration of compounds into select intracellular environment can influence the efficacy of a drug by restricting its interaction with the target site. Zhang et al (2003) suggest that modification of these ABC transporters may effectively improve the efficacy of compounds to CNS and target site. These functional transporters consist of two transmembrane domains and two nucleotide binding domains that contain the Walker A, Walker B and ABC signature motifs and are involved in the transport of substrates across membranes via the ATP energy-driven process (Lage et al.2003; Buss et al. 2002; Poretta et al. 2008). The over-expression of some members of this protein family, such as P-glycoproteins (P-gp/MDR/ABCB1) has caused multidrug resistance due to a decrease drug concentration to the target site (Lespine et al. 2012). However, very limited information is reported regarding ABC transporter interaction in mosquitoes; the majority is in humans, nematodes, or bacteria.

ABC transporters have been found in several insect species such as mosquitoes, tobacco hornworm, and fruit flies to provide a defense against insecticides (Buss *et al*., 2002; Bariami et al. 2012; Broehan et al. 2013; Figueira et al. 2014; Porretta *et al*., 2008). The interaction of ABC transporters inhibitors such as verapamil or ivermectin with insecticides hase been shown to have an increased in insecticide toxicity in resistant insect strains with over-expressed ABC transporters (Buss *et al*., 2002; Figueria et al. 2014; Porretta *et al*., 2008). Classic MDR modulators, including verapamil, quinidine, ivermectin, and cyclosporin A, are known inhibitors of the MDR phenotype. These ABC transporters inhibitors involve blocking drug efflux as a result of competition between modulators and transport substrates including the basal ATPase activity of ABC transporters; therefore, they are widely used to understand the interactions of

compounds and other inhibitors within these transporters (Figueira et al. 2014). Demauw et al. (2013) report that these inhibitors bind to ABC transporters and inhibits its transport function. Thus, a better understanding of ABC transporters interaction with anticholinesterases is of critical importance for control of mosquito borne diseases.

This study examined the effects of tacrine, *bis*(7)-tacrine, and verapamil on the acetylcholinesterase and ABC transporter ATPase activity of the yellow fever mosquito, *Ae. aegypti*. Herein, I report the: 1) inhibition of acetylcholinesterase activity in mosquitoes treated tacrine and *bis*(7)-tacrine alone and in combination with verapamil and 2) the stimulatory and inhibitory effects of tacrine, *bis*(7)-tacrine, and verapamil on the ABC transporter ATPase activity of mosquitoes.

4.2 Materials and Methods

4.2.1 Chemicals

Bis(7)-tacrine, ethanol (EtOH), dimethyl sulfoxide (DMSO), magnesium chloride ($MgCl_2$) and tacrine- 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate ACROS organics (New Jersey) were purchased from Fisher Scientific (Pittsburg, PA). Acetylthiocholine iodide (ATCh), adenosine 5'triphosphate disodium salt (ATP-Na), 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), Ethylene glycol-bis(2-aminoethylether)-N,N,N'N'-tetraacetic acid(EGTA), n-phenylthiourea, protease inhibitor cocktail, potassium nitrate (KNO_3), phenylmethylsulfonyl fluoride (PMSF), sodium azide, sodium dodecyl sulphate (SDS), thapsigargin, trizma base (Tris hydrochloride, Tris-HCl), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Ouabain was purchased from Chem Service (West Chester, PA). Verapamil was purchased from INDOFINE

Chemical Company, Inc. (Hillsborough, NJ). Cyclosporin A, dexamethasone, erythromycin, ketoconazole, nifedipine, paclitaxel, quinidine, vinblastine were purchase from Sigma-Aldrich (St. Louis, MO).

4.2.2 In Vivo Inhibition of Acetylcholinesterase Activity

A study of acetylcholinesterase (AChE) activity in 3-5 d old adults female mosquitoes treated with tacrine, *bis(*-7)-tacrine, and verapamil was conducted according to the procedures described by Anderson et al. (2009) with slight modifications. Adult mosquitoes were exposed to tacrine (10 µg/mosquito) or *bis(*-7)-tacrine (5 µg/mosquito) at the solubility limit individually and in combination with a fixed dose of verapamil (20 µg/mosquito). After twenty four hours, mosquitoes were dissected into head, thorax + abdomen, and whole body (head + thorax + abdomen) dissections. Each sample was homogenized in ice-cold 0.1 M sodium phosphate (pH 7.8) containing 0.3% Triton X-100 (v/v) at the rate of one hundredadult per 100 µl sodium phosphate. The individual homogenates were centrifuged at 10,000 x g for 10 min at 4 °C and the supernatant was used as the enzyme source for measuring AChE activity. The residual AChE activity in the supernatant was measured using a SpectraMax M2 multimode microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm immediately after the addition of acetylthiocholine iodide (ATChI) and 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB). The final concentrations of ATChI and DTNB were 0.4 mM and 0.3 mM, respectively.

4.2.3 In Vitro Activity of ATPase Synthases

The ABC transporter ATP synthase (ATPase) activity of 3-5 d old adults female mosquitoes treated with tacrine, *bis*-7-tacrine, and verapamil at 0.5 mM was examined using similar

procedures described by Figueria et al. (2013) with slight modifications. Adult mosquitoes were homogenized in ice-cold buffer containing 70% solution A (3 mM ATP-Na, 4 mM $MgCl_2$, 1 mM EGTA, 100 mM Tris-HCL (pH 7.5), 0.5 mM ouabain, 0.01 mM thapsigargin, 1 mM sodium azide, and 50 mM KNO_3) and 30% solution B (protease inhibitor cocktail, 3.3 mM PMSF, 4.8 mM EGTA, and 1 mM phenyl thiourea) at the rate of one adult per 1 µl buffer. The individual homogenates were centrifuged at 10,000 x g for 10 min at 4 °C and the supernatant was used as the enzyme source for measuring ATPase activity. The supernatants were incubated for 1 h at 37 °C with one concentrations of tacrine, *bis*-7-tacrine, and verapamil prepared in 1% DMSO. The reaction was stopped with the addition of 250 µl of ice-cold 1% SDS. The residual ATPase activity in the supernatant was measured using a SpectraMax M2 multimode microplate reader (Molecular Devices) at 390 nm. In order to measure ATPase activity using a human ABC transporter, the P-gp-Glo™ Assay System was used to detect changes in luminescence in a human cell line with select compounds. Cyclosporin A, dexamethasone, erythromycin, ketoconazole, nifedipine, paclitaxel, quinidine, vinblastine were diluted into a P-gp-Glo™ assay buffer to a final concentration of 0.5 mM. The ATPase activity was measured using a Promega GloMax 96 Microplate Luminometer.

4.2.4 Measurement of Total Protein Content

The total protein content in each sample preparation was determined using a bicinchoninic acid assay as described by Smith et al. (1985) with bovine serum albumin (Sigma Aldrich) as a standard. Mosquitoes were collected and homogenized in ice-cold 0.1 M sodium phosphate buffer (pH 7.8) containing 0.3%

Triton X-100 (Sigma Aldrich, St. Louis, MO). The individual homogenates were centrifuged at 10,000 x g for 10 min at 4 ˚C and the supernatant was used as the protein source for measuring protein content. Mosquito homogenates were incubated with a bicinchoninic acid (BCA) (Sigma Aldrich) plus 4% cupric sulfate solution at 37˚C for 30 min, after which time protein measurements were performed on a multimode microplate reader at 560 nm.

4.2.5 Statistical Analysis

The change in relative luminescence units (RLU) was calculated by subtracting the RLU of the test compound from the RLU of the untreated samples (Na_3VO_4) to reflect change in ATPase activity. A one-way analysis of variance in combination with a Tukey's multiple comparison test was used on the acetylcholinesterase and Dunnett's multiple comparison test was used on ABC transporter ATPase activity to compare the effects of tacrine, *bis*(7)-tacrine, and verapamil of the mosquitoes (GraphPad, La Jolla, CA).

4.3 Results

4.3.1 In vivo Inhibition of Acetylcholinesterase Activity

The residual activity of AChE in the head, thorax + abdomen, and head + thorax + abdomen of adult mosquitoes treated with tacrine (5 µg/mosquito) and *bis*(7)-tacrine (5 µg/mosquito) alone and in combination with verapamil (20 µg/mosquito) is shown in Figure 2. The AChE activity in mosquito heads treated with a combination of tacrine and verapamil was significantly reduced by 44.7% compared to the tacrine-only treatment (Fig. 2). However, there was no significant differences in AChE activity between the thorax + abdomen or head + thorax + abdomen of

mosquitoes treated with tacrine alone or in combination with verapamil. Similarly, the AChE activity in mosquito head treated with a combination of *bis*(7)-tacrine and verapamil was significantly reduced by 70.6% compared to the *bis*(7)-tacrine-only treatment (Fig. 2). Again, there was no significant differences in AChE activity between the thorax + abdomen or head + thorax + abdomen of mosquitoes treated with *bis*(7)-tacrine alone or in combination with verapamil.

4.3.2 In vitro Analysis of ABC Transporter ATPase Activity

The ABC transporter ATPase activity in adult mosquitoes treated with verapamil (20 µg/mosquito), tacrine (5 µg/mosquito), and *bis*(7)-tacrine (5 µg/mosquito) is shown in Figure 3. The ATPase activity of mosquitoes treated with verapamil, tacrine, and *bis*(7)-tacrine was significantly increased by 1.9-, 8.1-, and 8.1-fold, respectively, compared to the basal ATPase activity of the untreated mosquitoes (Fig. 3). The ATPase activity of a human ABC transporter expressed in a cell line treated with class I (i.e., the concentration-dependent activation of ATPase activity observed at low concentrations and inhibition at higher concentrations) and II ABC transporter (i.e., the concentration-dependent activation of ATPase activity) substrates is shown in Figure 3. The ATPase activity of the human ABC transporter treated with the class I substrate ketoconazole was significantly increased by 2.1-fold whereas cyclosporin A significantly decreased ATPase activity by 2.9-fold compared to the basal ATPase activity (Fig. 3). The class II substrates erythromycin and nifidipine as well as verapamil significantly decrease the ATPase activity of the human ABC transporter by 2.4-, 2.3-, and 1.3-fold, respectively, compared to the basal ATPase activity (Fig. 3). Both tacrine and *bis*(7)-tacrine

significantly increase the ATPase activity of the human ABC transporter by 1.1- and 1.3-fold, respectively, compared to the basal ATPase activity (Fig. 3).

4.4 Discussion

This data gathered in this study suggests that the altered toxicity of tacrine and *bis*(7)-tacrine by verapamil might be associated with the ABC transporter interference of these anticholinesterases to their intended target site. The AChE activity in *Ae. aegypti* whole- and thorax and abdomen-body dissections was not significantly lower for tacrine and *bis*(7)-tacrine with verapamil than tacrine or *bis*(7)-tacrine only treatments. This result could be due to the chemical release during preparations of the homogenate. Tacrine and *bis*(7)-tacrine reduce AChE activity in the mosquito heads by *ca*. 13% and 3%, respectively, compared to control treatments; however, the addition of verapamil significantly increases the anticholinesterase activity of tacrine and *bis*(7)-tacrine by *ca*. 45% and 71% respectively, compared to tacrine and *bis*(7)-tacrine-only treatments (Fig. 2). Therefore, the enhanced inhibitions of AChE by the acetycholinesterase inhibitors may be the result of verapamil's effect on modulating ABC transporter activities. This indicates that poor blood brain barrier penetration could be due to a possible resistance mechanism by mosquito ABC transporter to transport out substrates and non-substrates.

This current study shows that tacrine and *bis*(7)-tacrine increase ATPase activity in *Aedes aegypti* at lower concentrations, compared to that of verapamil (Fig. 3). A previous study has demonstrated that suitable substrates such as verapamil are capable of facilitating reduced ATPase activity across the ABC transporters through blocking of active ATP sites (Liu et. al 2003). The differential increase in ATPase activity suggests that tacrine and *bis*(7)-tacrine are

more suitable substrates for ABC transporter compared to verapamil, consistent with results found in previous studies (Bariami et al. 2012; Broehan et al. 2013; Figueira et al. 2013,).Thus provide putative evidence that ABC transporters are a pharmacological obstacle to the delivery of these anticholinesterases to their intended target site. This suggest that ATP-binding cassette transporter positioned within the blood brain barrier could be involved in the BBB penetration issue of target site delivery of tacrines to the central nervous system. These data are in alignment with our previous studies that have confirmed the blood-brain barrier of *Drosophila melanogaster* to be a limiting factor in tacrine anticholinesterase toxicity (Mutunga et al. 2012).

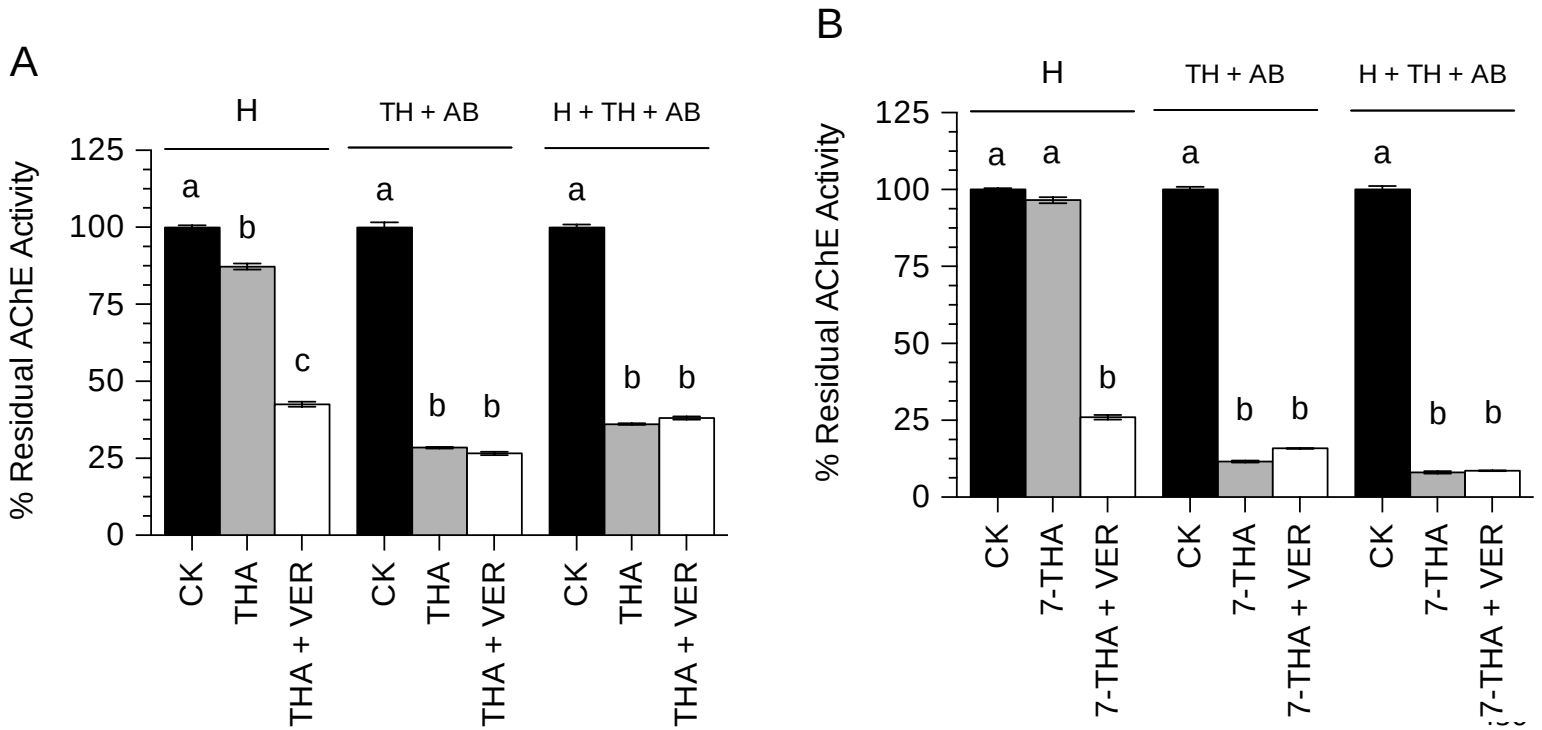

Figure 2. Residual acetylcholinesterase (AChE) activity of dissected adult mosquitoes, *Ae. aegypti*, exposed to A) tacrine or B) *bis*(7)-tacrine (AChE inhibitors) alone or in combination with verapamil (ABC transporter inhibitor). Tacrine (THA) or *bis*(7)-tacrine (7-THA) was topically administered at 10 µg or 5 µg, respectively, alone or in combination with verapamil (VER) at 20 µg for 24 h. Vertical bars indicate standard errors of the mean (n = 4). Asterisks on the bars indicate that the means are significantly different among the treatments for each dissection using a one-way analysis of variance and Tukey's multiple comparison test ($p < 0.05$). CK = control, H = head dissection, TH + AB = throax and abdomen dissection, H + TH + AB = whole body.

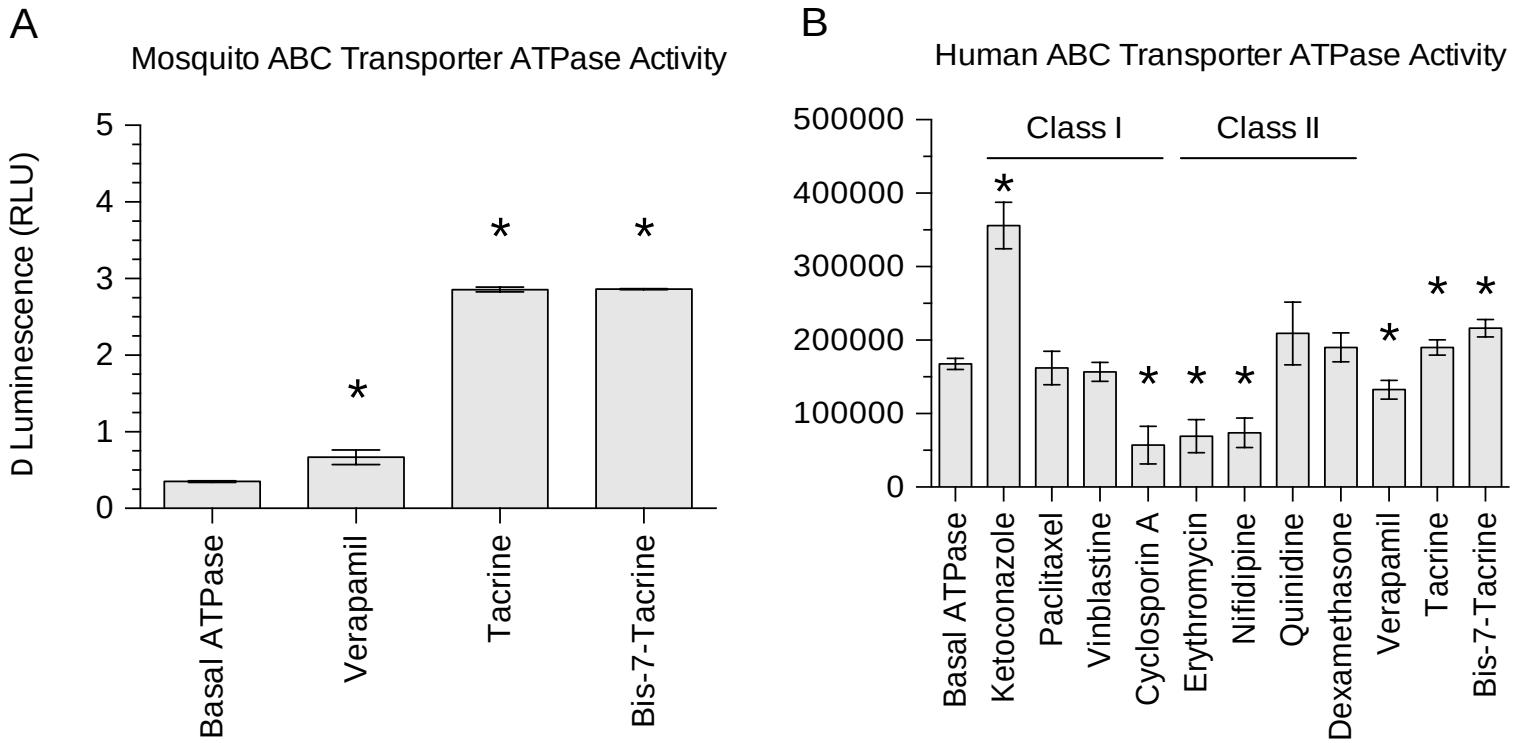

Figure 3. Luminescence measurement of adult mosquitoes, *Ae. aegypti*, and human ATPase activity treated with ABC transporter substrates. A) Mosquitoes were treated with 0.5µM verapamil, tacrine, or *bis*(7)-tacrine. B) Human ABC transporter cell line was treated with verapamil, tacrine, or *bis*(7)-tacrine as well as class I (ketoconzaole, paclitaxel, cinblastine, cyclosporin A) and II (erythromycin, nifidipine, quinidine, dexamethasone, verapamil) ABC transporter substrates at 0.5 mM concentrations. Vertical bars indicate standard errors of the mean (n = 4). Asterisks on the bars indicate that the means are significantly different among the basal treatments (control = Basal ATPase, untreated) using Dunnett's multiple comparison test ($p < 0.05$).

CHAPTER 5

SUMMARY

The specific aim of this study was to evaluate the ABC transporter interaction to impede the ability of tacrine-based compounds to reach the target site in the yellow fever mosquito, *Aedes aegypti*. The anticholinesterase inhibitors tacrine and *bis*(7)-tacrine and verapamil were used in this study. The mosquitoes were first exposed to tacrine alone and in combination with verapamil to examine the ABC transporters inhibitor (verapamil) interaction to the acute toxicities. Second, the acetylcholinesterase and ATPase activities were used to examine the metabolic detoxification and substrate favorability in the adult mosquito.

Acetylcholinesterase are enzymes that are able to hydrolyze acetylcholine in the central nervous system resulting in the buildup of ACh in the nerve synapse and resulting in causing death (Anderson et al. 2009). Tacrine and *bis*(7)-tacrine are experimental AChE inhibitors and verapamil is a known ABC transporter inhibitor. The literature suggests that metabolic and target site resistance limits anticholinesterases their ability to reach their intended target site resulting in a decrease in toxicity (Mutunga et al. 2013).

The goal of this study was to examine the acute toxicities of tacrine and *bis(*7)-tacrine alone and in combination of verapamil to *Ae. aegypti* adult as well as acetylcholinesterase and ATPase activities of the adult exposure to verapamil. The data gathered in this study provides evidence for insecticide transport substrates (tacrine and *bis*(7)-tacrine) and inhibitory ligands (verapamil) in *Ae. aegypti* adult may alter the toxic action of anticholinesterases to their target site within the

mosquito central nervous system. The overall hypothesis behind this proposed research is that ABC transporters impede the ability of tacrine-based compounds to inhibit AChE and, thus, reduce the toxicity of these anticholinesterases to *Ae. aegypti* adult.

This study demonstrates that adult treated tacrine, bis(7)-tacrine, and verapamil (ABC transporter inhibitor) were not acutely toxic to mosquitoWhen combined with compounds such as verapamil, tacrine and *bis*(7)-tacrine toxicity increased in *Ae. aegypti* adult. An increased of mosquitoes to toxicity when exposed to verapamil, along with an increase in mortality as verapamil dose increases suggest that verapamil inhibits ABC transporters when applied in combination with tacrine and *bis*(7)-tacrine to bypass the BBB in adult mosquito and, in turn, reach the target site. Thus, these data are in alignment with our previous studies that have confirmed the blood-brain barrier of *Drosophila melanogaster* to be a limiting factor in tacrine anticholinesterase toxicity (Mutunga et al. 2012).

The second objective of this study was to examine the acetylcholinesterase and ATPase activities in order to understand the metabolic detoxification and substrate favorability in the adult mosquito. I observed a decrease in residual anticholinesterase activity within mosquitoes exposed to tacrine or *bis*(7)-tacrine in combination with verapamil. In addition, our results indicated that tacrine and *bis*(7)-tacrine increase ATPase activity in *Ae. aegypti* at lower concentrations compared to that of verapamil. These results suggest that ABC transporters play a role in the transport of insecticides to the intended target site and a blood-brain barrier is a limiting faction in tacrine anticholinesterase toxicity, aligning with results found by Mutunga et al. (2013). However, additional experiments focused on the molecular characterization of

500 mosquito ABC transporters in *Ae. aegypti* to understand ABC transporter interactions and
501 resistance mechanism against insecticides. These results also suggest that tacrine and *bis*(7)-
502 tacrine are more suitable substrates for ABC transporters compared to verapamil and, thus,
503 provide evidence that ABC transporters is a pharmacological obstacle to the delivery of these
504 anticholinesterases to their intended target site and plays a role in insecticide resistance similar to
505 the one observed in the MDR phenotype. These results are similar to those found in other
506 studies (Demauw et al. 2014; Lage et al. 2012).
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