DETERMINATION OF ALLOSTERIC SOLVENT EFFECTS BETWEEN ACETYLCHOLINESTERASE AND MOSQUITO SELECTIVE CARBAMATES: IMPLICATIONS FOR HIGH THROUGHPUT SCREENING OF INSECTICIDES

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ABSTRACT

Malaria is vectored by the mosquito *Anopheles gambiae (Ag)* in Sub-Saharan Africa and infects approximately 500 million people annually. The increasing prevalence of pyrethroid-resistant mosquitoes has amplified the need for development of new, selective mosquitocides for use on insecticide-treated nets.

We have developed several phenyl-substituted *N*-methylcarbamates producing a high degree of selectivity for *Anopheles gambiae* acetylcholinesterase (*Ag*AChE) over human AChE. Molecular models suggest alternate conformations (flexibility) of W84 and W431 (*Ag* numbering) at the hydrophobic subpocket of the *Ag*AChE active site and poor flexibility within human AChE, allowing for the high selectivity of our novel carbamates. Initial selectivity data was obtained through screening of these insecticides while using ethanol as a solvent. Re-screening of these carbamates in the presence of 0.1% DMSO (*v/v*) resulted in antagonism of inhibition for *Ag*AChE, thus reducing the *Ag*AChE-selectivity by at least 10-fold. However, the presence of 0.1% DMSO did not antagonize the inhibition of human, *Drosophila melanogaster,* or *Musca domestica* AChE. Non-selective carbamates also displayed no solvent-dependent antagonism of inhibition in any species studied, including *Ag*AChE.

Molecular models provide an explanation for antagonism of inhibition when DMSO is present. I, and collaborators, propose that W84 and W431 in *Ag*AChE comprise an allosteric pocket that is stabilized by DMSO and is responsible for the solvent-dependent antagonism of inhibition observed with *Ag*AChE.

Dedication

I would first and foremost like to thank my family (Mom, Dad, Adam, Emily, and Katelyn) for their encouragement and steadfast support throughout my journey at Virginia Tech. 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 Virginia Tech. To my committee members, Drs. Sally Paulson and Don Mullins, thank you for your helpful guidance throughout my career.

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LITERATURE REVIEW

Mosquito borne vectors

Vector borne diseases are either emerging or resurging due to many different factors including, but not limited to, changes in 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 vectors 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 elimination of breeding sites and controlled insecticide usage (Gubler, 1998a). Malaria was greatly reduced in the Americas, Asia, and the Pacific Islands through control programs such as 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, and inhabitance of many ecosystems, scientists have deemed mosquitoes to be the most important arthropod affecting human health. For example, malaria is vectored by the mosquito species, *Anopheles gambiae* (Giles) and is regarded as the world's most important parasitic infection in humans (White, 1996). Despite rigorous control efforts, over 100 countries are at risk for malaria and it remains

the major cause of mortality in third world countries, with 1.5 to 3 million deaths per year. (WHO, 2003).

Aedes aegypti (Linn.) and *Aedes 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). 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). *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, *Aedes* species have been found to transmit various other diseases such as West Nile Virus, LaCrosse encephalitis, and Yellow Fever (WHO, 1997; Nash et al., 2001; Watts et al., 1973).

Other mosquito genera, such as *Culex*, are also known to act as vectors for numerous other diseases that affect thousands of people worldwide. *Cx. quinquefasciatus* (Say) is debatably the largest 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 ubiquitous presence of mosquitoes, control programs are vital for reduction of disease.

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 production of dichloro-diphenyl-trichloroethane (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). Although larvicides are still used, we currently implement a combination of control methods including synthetic pesticides, environmental management, and biological control. 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). However, 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 (Turusov et al., 2002; Roberts, 1997). This ban has resulted in researchers attempting to develop mosquitocides that will be persistent, selective, possess minimal side effects, and cheap to produce (Carlier et al., 2008; Berg, 2009).

The WHO has focused on malaria reduction in sub-Saharan Africa by controlling the vector with the use of two primary methods. IRS has been utilized by spraying the house interior

and eaves of houses with a persistent pesticide, such as DDT. Although utilization of IRS has had great success in decreasing the concentration of malarial vectors, there has been a decline 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, 2007). There have been several studies which reported reduced malaria infection due to reduction of insect vectors through the use of ITNs (Choi et al., 1995; Curtis et al., 1998). In the United States, malaria has been eradicated, even though the vector is still present. This eradication was achieved through the use of synthetic pesticides, and through improved socio-economic conditions such as window screens/air conditioners (Williams, 1963; Zucker, 1996).

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 site for *Aedes* spp., which has implications for control measures (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* other botanical larvicides (Kumar et al., 2009; Borovsky, 2003). However, prolonged use of several aforementioned synthetic larvicides has lead 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 potentially increase resistance. Due to these factors, there is a need for the design of new, selective mosquitocides for the control of disease vectors.

Another important mosquito vectored-disease is lymphatic filariasis, transmitted by *Cx. quinquefasciatus*. Control of this disease is based around reduction of mosquito numbers, but also incorporates treatment of the microfilaria through 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 on *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 currently enjoys success, as permethrin-incorporated Olyset Net® bed nets increased exophily of *Cx. quinquefasciatus* by 14%, and 15% completed a successful blood meal with a 'new' Olyset Net, compared to 35% with untreated nets (Guessan et al., 2008). As with other mosquito-vectored diseases, there is a need for new, selective mosquitocides for continued reduction of lymphatic filariasis.

Insecticides for vector control

The use of insecticides for mosquito control remains the most important and effective component of the integrated vector management approach for the control of vector borne diseases (Hemingway and Ranson, 2000). Insecticides have been developed with a variety of mechanisms and target sites. For instance, pyrethroids affect voltage-gated sodium channels and are currently the leading insecticide utilized in ITNs (Bloomquist, 1999). Neonicotinoids, act as agonists of nicotinic acetylcholine receptors (*n*AChR's) and several acetylcholine (ACh) mimics are available to control chewing and sucking agricultural pests (Bloomquist, 1999). Chlorinated cyclodienes act as GABA antagonists blocking chloride channels (Bloomquist, 1999). Acetylcholinesterase (AChE) inhibitors block the hydrolytic action of AChE, and carbamates (CB) and organophosphates (OP) are two classes of insecticides commonly known to inhibit this enzyme (Bloomquist, 1999). Fig. 1.1 depicts common CB's (Bendiocarb and Propoxur) and OP's (Malathion and Fonofos) used for vector control.

The insecticidal effect of carbamates and organophosphates is due to their inherent ability to inhibit acetylcholinesterase. AChE is a serine hydrolase which is needed for regulating the synaptic action of the neurotransmitter, acetylcholine. The AChE-directed insecticides react

with a serine residue that is located at the catalytic site found within the AChE gorge (Fukuto, 1990). The carbamylated or phosporylated enzyme is no longer able to hydrolyze acetylcholine (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, 2002). Although many OP's and CB's have a high insect toxicity, their mode of action allows for minimal selectivity. Both mammalian and insect AChE possess a serine residue at the catalytic site within the AChE gorge, causing poor selectivity and thereby limiting the use of many AChE inhibitors (Pang, 2009).

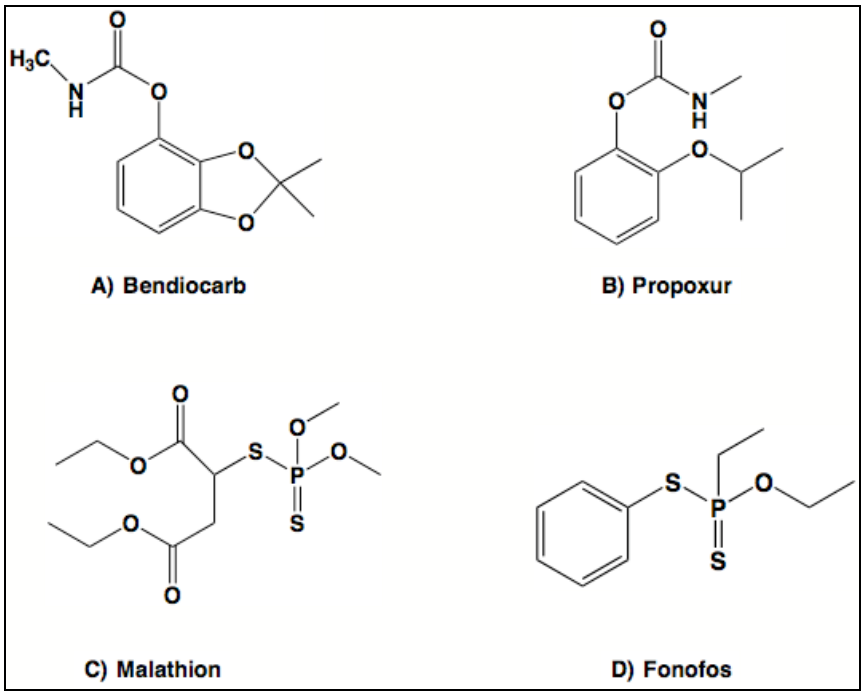

Fig. 1.1. Two commonly used carbamates (A, B) and organophosphates (C, D) for controlling disease carrying mosquito vectors

Insecticide Resistance

Insecticide resistance has become a major problem for controlling mosquitoes due to a continuous selection for resistance to nearly all deployed insecticides (Pasteur and Raymond, 1996). There are a variety of resistance mechanisms that have been identified and each must be

dealt with in vector borne disease control. Enzyme systems are involved in metabolism of xenobiotics, therefore leading to development of resistance to insecticides. For example, in *An. gambiae,* elevated levels of glutathione-*S*-transferases and cytochrome P450 monooxygenases (MFO) have been shown to cause DDT and pyrethroid resistance, respectively (Ranson et al., 2001, Chandre et al., 1999). Resistance to CB, OP, and pyrethroid insecticides can also be due to detoxication by elevated carboxylesterase levels (McCarroll et al., 2000). Single nucleotide mutations in the sodium channel gene are associated with knockdown resistance (*kdr*) to pyrethroids (Chandre et al., 1999). MACE (Modified AcetylCholinEsterase) is also a common resistance mechanism among insects, including *An. gambiae*. MACE is a target site mechanism that produces high resistance ratios toward dimethylcarbamates, such as pirimicarb (Foster et al., 2003).

The nearly uniform pattern of insecticide use to control dengue fever vectors has also increased the levels of resistance in *Ae. aegypti* (Rawlins, 1998). For nearly twenty years, temephos has been used as a larvicide and malathion has been used as an adulticide. The increased usage of these two compounds has caused target site resistance or metabolic resistance within this vector (Rawlins, 1998). As with *An. gambiae*, *Ae. aegypti* has expressed metabolic resistance through increased levels of α and β esterases corresponding to OP and CB resistance, and elevated MFO levels to yield pyrethroid resistance (Flores et al., 2006). Target site resistance has also been observed in *Ae. aegypti*. Additionally, there is reduced genetic variability and gene deletion found on chromosome 1 due to OP insecticide selection (Yan, 1998).

Insecticide resistance of mosquitoes due to agricultural uses has been documented and specifically effects insecticide design for disease control. Urban agriculture in Benin combined

with the use of broad spectrum insecticides on food plots has been linked to *An. gambiae* resistance of permethrin through a *kdr* mutation (Yadouleton et al., 2009). As shown above, 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), so a balance of uses may need to be established in an area. For instance, in locations of disease control, one should limit or manage (i.e., alternate mode of action) agricultural pesticide usage to prevent cross-exposure resistance. Development of a highly selective insecticide with poor toxicity to agricultural pests, and therefore less ancillary uses, can mitigate resistance due to limited selection pressure within breeding sites.

Development of new anticholinesterase inhibitors

The current problems associated with insecticide use 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* (*Tc*AChE) 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 for the synthesis of a library of anticholinesterases compounds (Bourne et al., 2004; Bartolucci et al., 2001). 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 structure and function of other insect AChE gorge structures through comparative molecular

modeling, bearing in mind that there are two *ace* genes in insects and the *Drosophila* crystal structure belongs to *ace-2*.

Within the AChE gorge there is a peripheral site, located near the mouth, a narrow portion (known as the "bottleneck") located about halfway down the gorge, the catalytic acyl site, found at the bottom of the gorge, and anionic site located adjacent to the acyl site (Pang, 2006; Botti et al., 1999). A depiction of this AChE gorge structure is shown in Fig. 1.2.

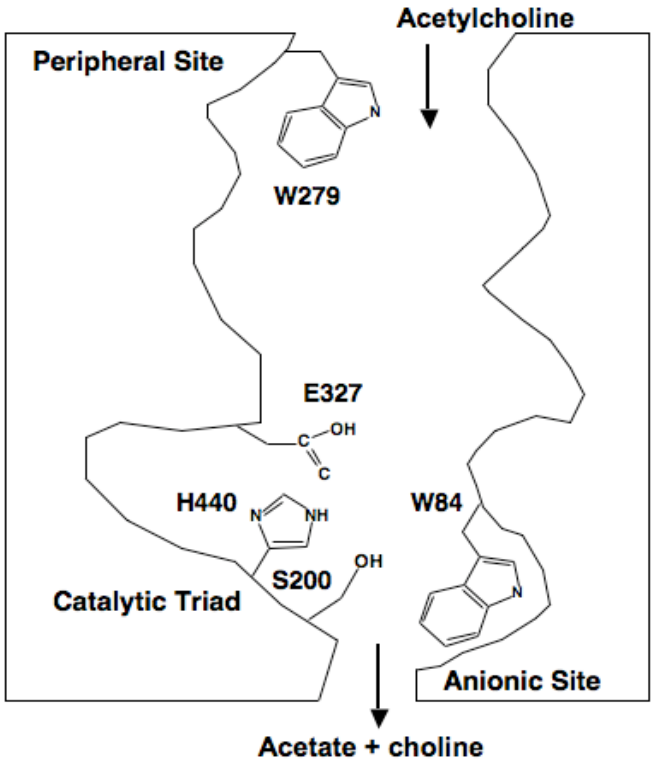

Figure 1.2. Diagram of *Ag*AChE gorge showing some of the relevant amino acid residues at the peripheral (W279), anionic site (W84), and catalytic acyl sites (S200). Designed by JR Bloomquist.

Molecular models suggest that the peripheral and active sites of *Ag*AChE and human AChE (*h*AChE) consist of differing and unique amino acids, which can assist in the design of a selective carbamate (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 is currently being studied through site directed mutagenesis. Through

utilization of these models, design of selective compounds that are capable of interacting with key amino acids is possible. Fig. 1.3 shows experimental compounds that demonstrated high potency and selectivity to *An. gambiae* AChE (*Ag*AChE) (Carlier et al., 2008) and will be used in this study. Figure 1.4 displays PRC 521, a non-selective experimental carbamate.

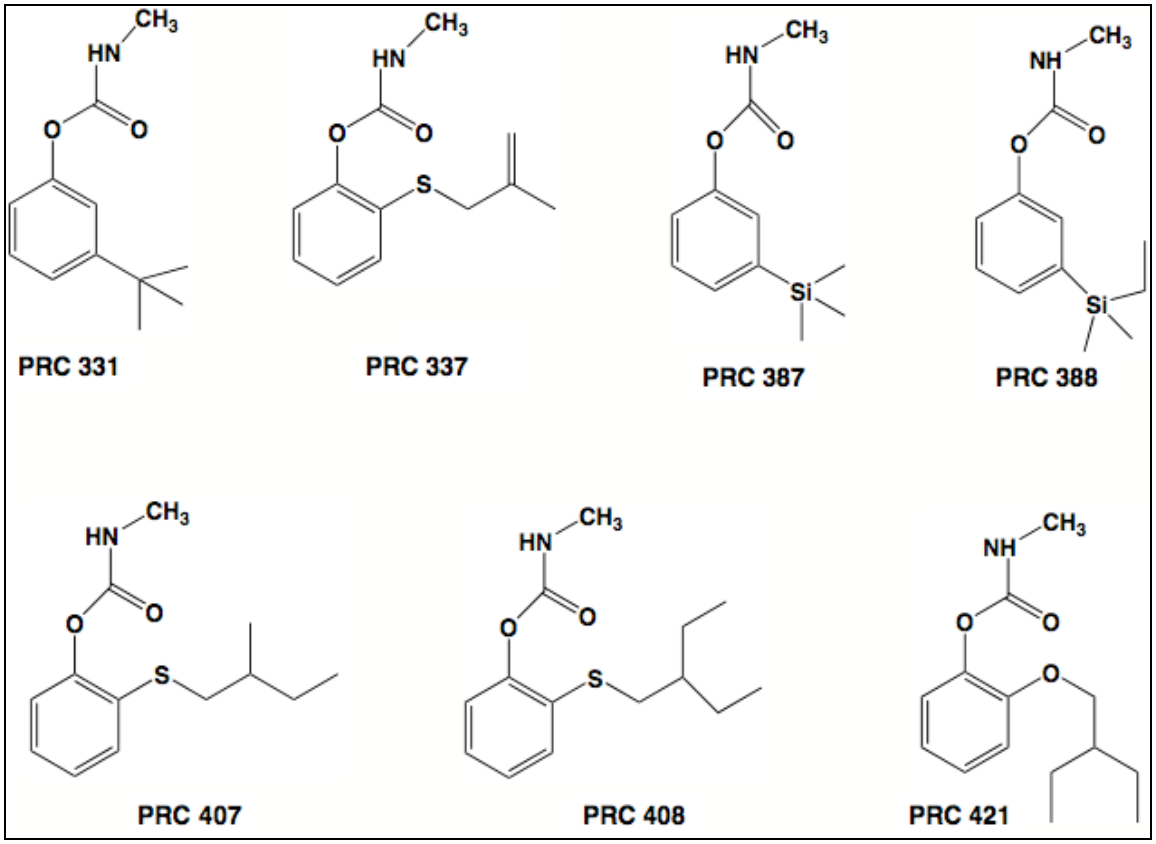

Fig. 1.3. Highly selective and species-sensitive carbamate molecules designed for use on *Anopheles gambiae*, and referred to in this study. IUPAC names of the experimental compounds are: 3-(*t*-butyl)phenyl methylcarbamate (Terbam, Knockbal, TBPMC, PRC 331), 3-(ethyldimethylsilyl)phenyl methylcarbamate (PRC 337), 3-(trimethylsilyl) phenyl methylcarbamate (PRC 387), 3-(ethyldimethylsilyl)phenyl methylcarbamate (PRC 388), 2-(2methylbutylthio)phenyl methylcarbamate (PRC 407), 2-(2ethylbutylthio)phenyl methylcarbamate (PRC 408), and 2-(2-ethylbutoxy)phenyl methylcarbamate (PRC 421).

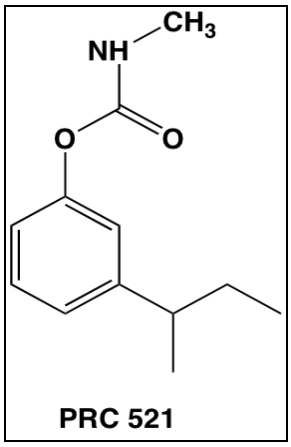

Figure 1.4. Non-selective experimental carbamate, PRC 521. IUPAC name: 3-(*sec-butyl*)phenyl methylcarbamate.

High-Throughput Screening of Insecticides

Due to the large demand for selective insecticides, chemical companies have developed high-throughput screening (HTS) processes for discovery of insecticides. HTS allows researchers to obtain lead compounds from chemical libraries through *in vitro* or *in silico* studies. 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* analysis. Research has shown DMSO can act as a stabilizer (Rajendran et al., 1995), denaturant

(Bhattacharjya and Balaram, 1997; Jacobson and Turner, 1980; Fujita et al., 1982; Kovrigin and Potekhin, 1997), inhibitor (Perlman and Wolff, 1968; Kleifield et al., 2000; Johannesson et al., 1997), 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, selectivity can be overlooked due to solvent-dependent antagonism during *in vitro* assays.

Model Organisms

Comparison of 3–D structures and homology models can help to explain differences in activity and specificity between different insect AChE enzymes (Harel et al., 2000). AChE crystal structures are important for studying the structure–function relationship of the enzyme and experimental compounds. *Drosophila melanogaster* AChE is the sole insect crystal structure documented and is needed for comparison to other insect AChE gorge structures (Harel et al., 2000).

Musca domestica, the housefly, was the model organism used to study resistance mechanism and to screen many anticholinesterases during the 1960's (Georghiou, 1965). It is also important to understand the relationship between the insecticidal compounds and other insects within the order of the target organism. *Musca domestica* and mosquitoes are both found in the order Diptera. Therefore, *M. domestica* can assist in determining if toxicity to diptera is uniform.

Overall Objective

The overlying goal of this project was to determine the interactions between seven selected experimental compounds (PRC 331, PRC 337, PRC 387, PRC 388, PRC 408, and PRC 421, PRC 521), DMSO, and acetylcholinesterase. The seven experimental compounds are shown in Figure 1.3 and six of the seven compounds were selected for their high degree of selectivity towards *An. gambiae* (Table 1.1), as shown from previous work (Anderson *et al.,* 2008). PRC 521 was selected for its high potency, but poor selectivity characteristics.

Table 1.1. Effects of *N*-methylcarbamate insecticides on *An. gambiae* and human AChE activity (Anderson et al., 2008).

	An. gambiae AChE			Human AChE			
Inhibitor	IC_{50}, nM; (95% CI)	Hill slope	r^2	IC_{50}, nM; (95% CI)	Hill slope	r^2	MS*
Propoxur	371 (320-421)	0.94	0.99	1710 (1420-2060)	1.31	0.99	5
PRC 331	3 (2-4)	0.50	0.99	265 (240-293)	0.98	0.99	88
PRC 337	124 (117-132)	0.84	0.99	9551 (7695-11850)	0.97	0.98	77
PRC 387	6 (3-9)	0.71	0.96	532 (375-756)	0.81	0.97	89
PRC 407	30 (25-36)	0.77	0.99	3543 (3152-3904)	0.95	0.99	118
PRC 408	3 (2-4)	0.86	0.98	3627 (3182-4134)	1.06	0.99	1204
PRC 421	276 (245-302)	0.96	0.99	98820 (89110-109600)	1.52	0.98	358
PRC 521	9 (7 – 12)	0.59	0.99	12 (8 – 16)	0.77	0.99	1.3

*Mosquito Selectivity = IC_{50} of Human AChE / IC_{50} of mosquito AChE

The high selectivity of these compounds separates them from standard carbamates, such as propoxur (3-fold selectivity), making them more marketable for production (Carlier et al., 2008). Development of valid HTS screens requires an understanding of solvent-enzyme interactions during *in vitro* colorimetric assays. Solvent-dependent antagonism will show a falsified decrease in selectivity due to variability of enzyme structure between the human and *Ag*AChE. This falsified result could potentially lead to rejection of potent and selective inhibitors during the high-throughput screening process.

Chapter 2

PRELIMINARY STUDIES

As previously mentioned, the need for selective insecticides for malaria control is of growing concern. Work in our laboratory has identified several carbamates that possess high selectivity of *An. gambiae* AChE (*Ag*AChE) vs. human AChE (*h*AChE), seen in Table 1.1. The high selectivity of these carbamates potentially allow for usage on insecticide-treated nets (ITN) within Sub-Saharan Africa to minimize malarial transmission. However, it is important to understand the enzyme potency and toxicity toward other insects, specifically agricultural pests.

Insecticide resistance of mosquitoes due to agricultural uses has been documented and specifically affects insecticide design for disease control. Urban agriculture in Benin combined with the use of broad spectrum insecticides on food plots has been linked to *An. gambiae* resistance toward permethrin through a *kdr* mutation (Yadouleton et al., 2009). As shown above, 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). Development of a highly selective insecticide with poor toxicity to agricultural pests can mitigate resistance due to limited selection pressure within breeding sites. Similarly, to increase compliance of ITN use, it is important to understand the toxicity toward nuisance biting mosquitoes and other disease vectors.

Data was originally collected to determine the enzyme potency and toxicity of our novel carbamates toward agricultural pests, mosquito vectors secondary to *An. gambiae,* and model organisms. However, rescreening of our chemical libraries with slight changes to the protocol yielded unexpected results on inhibition, thus reducing the *Ag*AChE vs. *h*AChE selectivity ratios. This phenomenon had to be explained to ensure the selectivity ratios described in table 1.1 were correct.

Chemical companies must screen thousands of compounds to determine those with high potency and selectivity. The process of screening chemical libraries is usually a high-throughput screen (such as a *in vitro* colorimetric or fluorometric assay) that has been optimized to facilitate quick identification of potent compounds. This "quick screen" usually utilizes one solvent and one protocol to identify compounds of interest and does not typically factor in relationships between compounds, solvent, and the protein.

As with pharmaceutical compounds, protocols in our laboratory were designed for high throughput screening of our chemical libraries. Due to chemical evidence of high solubility in ethanol or methanol, all compounds were originally screened using the aforementioned solvents with one protocol, which will be referred to as Protocol A. Protocol A dictated the inhibitor be suspended into a solvent of choice to create a 0.1 M stock solution. The stock solution was then diluted (100x) into 0.1 M sodium phosphate buffer and serial dilutions (10x) were then

performed using only buffer. This method allowed for a constant ratio of solvent to inhibitor. All original selectivity data was obtained utilizing protocol A.

Our experimental procedures were later standardized with dimethyl sulfoxide (DMSO) being the solvent of choice for all experiments. When using Protocol A, this solvent change did not alter the IC_{50} values that were previously found (Table 3.1). However, the data could not be replicated when Protocol A was slightly modified to maintain a constant percentage (0.1% (v/v)) of DMSO throughout the experiment, which will be referred to as Protocol C. This method also utilizes a 0.1 M inhibitor stock suspended in DMSO. Serial dilutions (10x) were performed in 100% DMSO. Each concentration was then diluted (100x) into 0.1 M sodium phosphate buffer to create a final percentage of 0.1 % DMSO. In Protocol C, the inhibitor was the sole independent variable versus a constant proportion of solvent/inhibitor seen in Protocol A. Protocol C drastically increased IC_{50} values in both mosquito enzymes when this alternate protocol was used and thus, decreased the selectivity ratios. For example, the IC50 value for PRC331 on *Ag* homogenate went from 3 nM (protocol A) to 113 nM (protocol C), a 37-fold, statistically significant increase. 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 *An. gambiae* AChE. Implications toward high throughput screening of insecticides are further discussed within.

Chapter 3

DETERMINATION OF ALLOSTERIC SOLVENT EFECTS BETWEEN ACETYLCHOLINESTERASE AND MOSQUITO SELECTIVE CARBAMATES: IMPLICATIONS FOR HIGH THROUGHPUT SCREENING OF INSECTICIDES

INTRODUCTION

Acetylcholinesterase (AChE: EC 3.1.1.7) is a target site for insecticides due to its vital role in nerve signal propagation (O'Brien, 1967). AChE is a highly conserved, serine hydrolase necessary for regulation of the neurotransmitter acetylcholine in both human and insect central nervous systems. Anticholinesterases, such as carbamates (CB) and organophosphates (OP), inhibit AChE through carbamylation or phosphorylation of the serine (S199 in *Anopheles gambiae*) hydroxyl group within the catalytic triad (Fukuto, 1990; Carlier et al., 2008). Significant inhibition of AChE results in death due to a buildup of acetylcholine (ACh) in the nerve synapse, thereby preventing subsequent nerve impulses (Fukuto, 1990; Cohen and Oosterbaan, 1963; Pang, 2006).

Comparative structural analysis of AChE between species is vital for development of anticholinesterase insecticides and for an understanding of interactions between the enzyme, solvent, and inhibitor. The malaria mosquito, *Anopheles gambiae*, AChE (*Ag*AChE) gorge is composed of a peripheral site at the mouth, a 'bottleneck' located halfway down the gorge and a catalytic acyl site at the bottom of a 20 Å deep narrow gorge (Pang, 2006; Weill et al., 2004; Carlier et al., 2008). Homology models have shown a number of unique differences between *Ag*AChE and human AChE (*h*AChE). For instance, Asp441/Tyr449 (*Ag*AChE/*h*AChE

numbering) is a major difference and will be discussed in detail within this manuscript (Hosea et al., 1996).

Selectivity is a major factor in the development of insecticides and is defined as increased enzyme affinity towards mosquito enzyme (or enzyme of interest) over human enzyme. The majority of anticholinesterases are non-selective (similar potency towards mosquito and human enzymes), limiting the use of CBs and OPs as insecticides (Hollingworth, 1971).

The need for novel insecticides is increasing rapidly due to resistance of vectors/pathogens towards insecticides and the banning of current compounds (Zaim and Guillet, 2002). However, it is becoming increasingly difficult to commercialize chemicals due to cost of production, strict environmental regulations, and various other hurdles within the developmental process. To increase the possibility of discovering novel compounds, there has been a transition from whole organism testing towards *in vitro* and/or *in silico* high throughput screens (HTS) (Ridley et al, 1998).

In vitro assays, such as colorimetric or fluorometric assays, are helpful in determining inhibitor potency on a well-validated target site and are also capable of screening hundreds of thousands of compounds annually. Compounds that display high potency within these colorimetric assays are then subjected to *in vivo* assays to determine the toxicity of the experimental compound. This high-throughput method allows researchers to screen large quantities of agrochemicals, but also has the inherent downfall of enzyme inhibition due to assay artifacts and not true binding of inhibitor. Secondly, HTS screens potentially overlook potent and selective compounds due to molecular interactions between protein, solvents, and/or inhibitor.

To decrease variability, researchers have attempted to standardize the HTS process in various ways, including standardization of solvents used. Dimethyl sulfoxide (DMSO) is a simple amphipathic molecule and is the primary solvent used for solubilization of chemical libraries for HTS (Busby et al., 1998). DMSO is considered the solvent of choice due to its good dissolving ability, low chemical reactivity, and low vapor pressure (Tjernberg et al., 2006). Since DMSO has become the standard vehicle for drug discovery, there has been a large amount of research on storage of compounds in DMSO stock solutions. Many of these articles discuss how prolonged storage can potentially cause compound instability, decreased potency through freeze/thaw techniques, and the effect of water absorption on compounds (Cheng et al. 2003; Kozikowski et al., 2003). Denaturation/catalysis of enzymes by organic solvents (DMSO, acetonitrile, etc.) is also well documented (Busby et al., 1998; Obregon et al., 2005; Chauret et al., 1997). While this knowledge is important, there has been little documentation on the critical aspect of the effects of DMSO towards proteins during *in vitro* analysis. DMSO can act as a stabilizer (Rajendran et al., 1995), denaturant (Bhattacharjya and Balaram, 1997; Jacobson and Turner, 1980; Fujita et al., 1982; Kovrigin and Potekhin, 1997), inhibitor (Perlman and Wolff, 1968; Kleifield et al., 2000; Johannesson et al., 1997), and an activator (Rammler, 1967). However, these experiments were performed at DMSO concentrations (10% - 70%), which are greater than those typically encountered in HTS, which are 0.1%-5% (*v*/*v*) (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, selectivity can be overlooked due to solvent-dependent antagonism during *in vitro* assays.

The objective of this present investigation was to determine the effects of organic solvents on selective carbamates identified in our lab and whether these solvent effects can mask

selectivity of novel insecticides through solvent-dependent antagonism of inhibition during *in vitro* assays.

MATERIALS AND METHODS

Inhibitors and solvents

Propoxur and bendiocarb were purchased from Sigma-Aldrich (St. Louis, MO, USA). Experimental carbamates were provided by the Virginia Tech Chemistry Department through our collaboration with Dr. Paul Carlier. They were prepared by *N*-methylcarbamoylation of the corresponding phenol group. All experimental compounds were purified by column chromatography and/or re-crystallization and are >95% pure by ^{1}H NMR analysis.

Dimethyl sulfoxide (DMSO, 99.9%) and ethanol (EtOH, 95%) were both purchased from Sigma-Aldrich. Acetylthiocholine (ATCh)(≥ 99% purity) and 5,5'-dithiobis-(2-nitro) benzoic acid (DTNB)(99% purity) were also purchased from Sigma (St. Louis, MO, USA).

Molecular sieve UOP type 3Å were purchased from Sigma (St. Louis, MO, USA) and was used to prevent water absorption within the DMSO stock. Fifty beads were added into the 100 mL stock solution. These sieves have a diameter of ~2 mm, a pore size of 3Å, and a water absorbing capacity of ≥ 15%.

Enzymes

Five enzymes were used in this study: *Anopheles gambiae* homogenate (G3 strain, Virginia Tech entomology), *Musca domestica* homogenate, *Drosophila melanogaster* homogenate, CBL (*Ag*AChE recombinant enzyme), and recombinant *h*AChE (lyophilized powder, Sigma C1682). Homogenate enzymes were prepared through homogenization of insects. Ten whole bodied adult female mosquitoes (or 10 whole bodied *D. melanogaster* /5 *M.*

domestica heads) were homogenized in 1 mL of ice cold sodium phosphate buffer (0.1 M sodium phosphate, pH 7.8, all enzyme preps contained 0.3 % (v/v) 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. CBL enzyme (recombinant *Ag*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 of Creative BioLabs (CBL, Creative Dynamics Inc., Port Jefferson Station, NY, USA). The expression vector pFASTBac and Sf9 insect cells were used. Note that in the full-length precursor protein, Swiss-Prot code ACES_ANOGA, the corresponding numbering is D162-D701. From infection of 1 liter of insect cell culture, soluble *rAg*AChE (ace-1) was expressed and purified up to 90% pure (0.25 mg yield), first with an anion exchange Q column, followed by a Ni^{2+}-NTA gravity column. Prior to use in assay, CBL was diluted 300-fold with phosphate + Triton x-100 buffer. Human AChE (*h*AChE) was purchased from Sigma was diluted 500x with phosphate + Triton x-100 buffer prior to assay.

Enzyme Inhibition Assays

Ten µL of enzyme solution was added to each well of the 96-well micro assay plate along with 10 µL of dissolved compound and 80 µL of ice-cold phosphate buffer. The plate was then 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 100 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). The instrument read samples at 405 nm at room temperature for 10 min.

The results were analyzed by nonlinear regression to determine IC_{50} values, 95% CI, hill slopes, and r^2 values using GraphPad Prism™ (GraphPad Software, San Diego, CA, USA). T nonlinear regression equation used was as follows:

Y = bottom + (Top – Bottom) / (1 + 10 ^((LogEC_{50} – x)*Hillslope));

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

Y starts at the top and goes to the bottom with a sigmoid shape.

Constraints were set at 0 and 100 for bottom and top, respectively. The IC_{50} values and the hill slopes were used to determine the response characteristics of each compound for each insect species and solvent.

Inhibitor preparation protocols

Three protocols were utilized throughout this study to determine the inherent effects of solvent on IC_{50} and hill slopes. **Protocol A:** 100-fold dilution of 0.1 M stock solution of inhibitor suspended in solvent (DMSO or EtOH) into phosphate buffer at pH 7.8. 10-fold serial dilutions were then performed in buffer allowing a constant ratio of inhibitor to % solvent. Final inhibitor concentration range used was 10^{-4} M to 10^{-11} M, with the highest solvent (DMSO or EtOH) content being 0.1% (*v*/*v*) at the highest inhibitor concentration used; **Protocol B:** Identical to protocol A with the only exception being the starting stock concentration is 10 mM versus 0.1 M. Final inhibitor concentration range used was 10^{-5} M to 10^{-12} M; **Protocol C:** 10-fold dilution of 0.1 M stock solution of inhibitor dissolved in solvent of choice (DMSO or EtOH) into the corresponding organic solvent. Ten-fold serial dilutions were performed in solvent to give a range of stock solutions, that were then further diluted (100-fold) in 0.1 M sodium phosphate

buffer. This resulted in a final 0.1% (*v/v*) constant solvent throughout the experiment, so that the inhibitor concentration was the only variable in the experiment.

The Ellman assay (Ellman et al., 1961) was also utilized to determine the interaction between the AChE enzyme and DMSO; however, the protocol was modified slightly from Carlier et al. (2008) in the manner that the solvent was used as the inhibitor. Solutions were made with solvent (DMSO or EtOH) 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. Three enzymes were used: recombinant human AChE, recombinant *Ag*AChE, and *An. gambiae* homogenate.

The midpoint for the DMSO-dependent antagonism of *Ag*AChE was determined with the method for Protocol C. However, DMSO concentrations were held constant at concentrations ranging from 10^{-5}% to 1%. CBL enzyme was utilized for this experiment because it produced the most consistent responses, when compared to *Ag* homogenate enzyme. Three replicates were performed for each data point (1 replicate IC_{50} value was determined from eight inhibitor concentrations, using nonlinear regression to determine IC_{50}).

Statistical analyses

IC_{50} values for each species were calculated by nonlinear regression using GraphPad Prism™ (GraphPad Software, San Diego, CA, USA). IC_{50} values for each protocol were compared by a one way ANOVA and Tukey's multiple comparison test. Error bars represent 95% confidence limits (CI). The DMSO effect on enzymes was determined by calculating the percent residual activity using the formula: (concentration OD value / control OD value)*100.

The mean (n = 3) percent residual activity was compared with a one way ANOVA and Tukey's multiple comparison test (Figure 3.8).

Molecular Modeling

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 *Ag*AChE was generated using Molsoft ICM (Abagyan et al., 1994). The X-ray structure of mouse AChE (mAChE, PDB 1D 1N5R) was used as a template for the *Ag*AChE catalytic subunit (D1-P540). The flexible peripheral site loop of *Dm*AChE was used as a template for initial modeling of the corresponding loop region of *Ag*AChE. Loop templates were extracted from the PDB and allowed us to further model the loop of *Ag*AChE, followed by Monte Carlo sampling of the side chains, and energy minimization of the backbone. The RMSD value of the backbone C atoms between the refined *Ag*AChE model and the *m*AChE template is 0.40 angstroms for 495 matches (Carlier et al., 2008).

RESULTS

DMSO-Dependent Antagonism of Ag*AChE*, h*AChE*, Dm*AChE*, *and* Md*AChE*

Comparison of *Ag*AChE IC_{50} values confirm there is little to no difference between IC_{50} values when using EtOH or DMSO as a solvent for experimental carbamates, when using Protocol A. The largest increase was PRC 331: IC_{50} increased from 3 nM to 10 nM (Table 3.1). Protocols A and B resulted in similar IC_{50} values for *Ag* homogenate and *Ag*AChE recombinant enzyme (CBL) when DMSO is used as the solvent, as shown in Figure 3.1. The largest difference between Protocols A and B for *Ag* homogenate was a 4-fold increase in IC_{50} (PRC

388) and a 6-fold increase in IC_{50} (PRC 331) from Protocol A to B on CBL. However, Figure 3.1 shows that the presence of constant 0.1% DMSO (*v/v*) resulted in a substantial decrease in inhibition (increased IC_{50}) for the highly selective experimental carbamates (PRC 331, PRC 337, PRC 387, PRC 388, PRC 408, and PRC 421; shown in Figure 1.3) among both *Ag*AChE enzymes. The increased IC_{50} values ranged from a 5-fold increase (PRC 421) to a 43-fold increase (PRC 388) on *Ag* homogenate. Similarly, increased IC_{50} values were observed on CBL and ranged from a 3-fold increase (PRC 337) to a 28–fold increase (PRC 331). However, non-selective carbamates displayed little statistical difference between the three protocols. From Protocols A to C, *Ag* homogenate displayed a mere 1.2 fold increase in IC_{50} value with propoxur and a 1.4 fold increase in IC_{50} value with bendiocarb. CBL displayed no statistical difference between IC_{50} values for propoxur, and bendiocarb displayed only a 1.4 fold increase in IC_{50} (Figure 3.1). A non-selective experimental carbamate (PRC 521; shown in Figure 1.4) displayed a 1.7 fold increase in IC_{50} value from Protocol A to Protocol C on *Ag* homogenate (Figure 3.2); a small increase relative to selective carbamates.

Table 3.1. Comparison of *An. gambiae* homogenate AChE inhibition when using EtOH as a solvent compared to using DMSO as a solvent.

	*Ag*AChE protocol A (EtOH)[a]			*Ag*AChE protocol A (DMSO)		
Inhibitor	IC_{50}, nM; (95% CI)	Hill Slope	r^2	IC_{50}, nM; (95% CI)	Hill Slope	r^2
Propoxur	371 (326-421)	0.94	0.99	296 (251-349)	0.9	0.99
PRC331	3 (2 - 4)	0.5	0.99	10.4 (8 - 13)	0.42	0.99
PRC 337	124 (117-132)	0.84	0.99	181 (162-199)	0.76	0.99
PRC 387	6 (3-9)	0.71	0.96	14 (9 - 16)	0.68	0.99
PRC 408	3 (2-4)	0.86	0.99	6 (5 - 7)	0.74	0.99
PRC 421	276 (248-307)	0.96	0.99	79 (51 - 121)	0.83	0.99

[a] Data set 1 was taken from Dr. Troy Anderson at VA Tech Entomology (Anderson et al., 2008.)

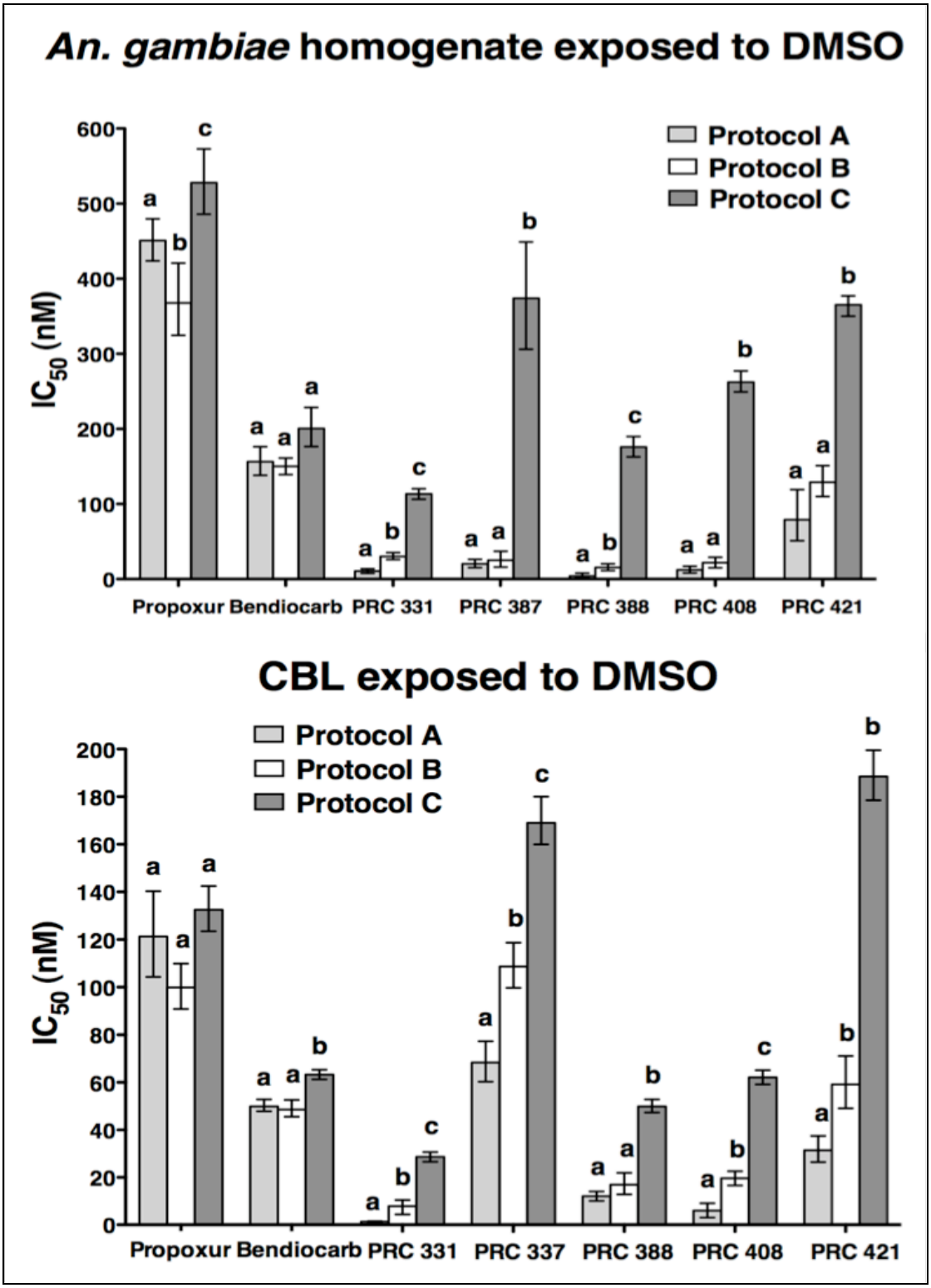

Figure 3.1. IC_{50} values for *An. gambiae* homogenate and CBL enzymes exposed to DMSO. Columns represent IC_{50} values from non-linear regression analysis and were compared by a one way ANOVA and Tukeys multiple comparison test. Bars not labeled by the same letters represent statistical significance at $P < 0.05$. Error bars represent 95% CI.

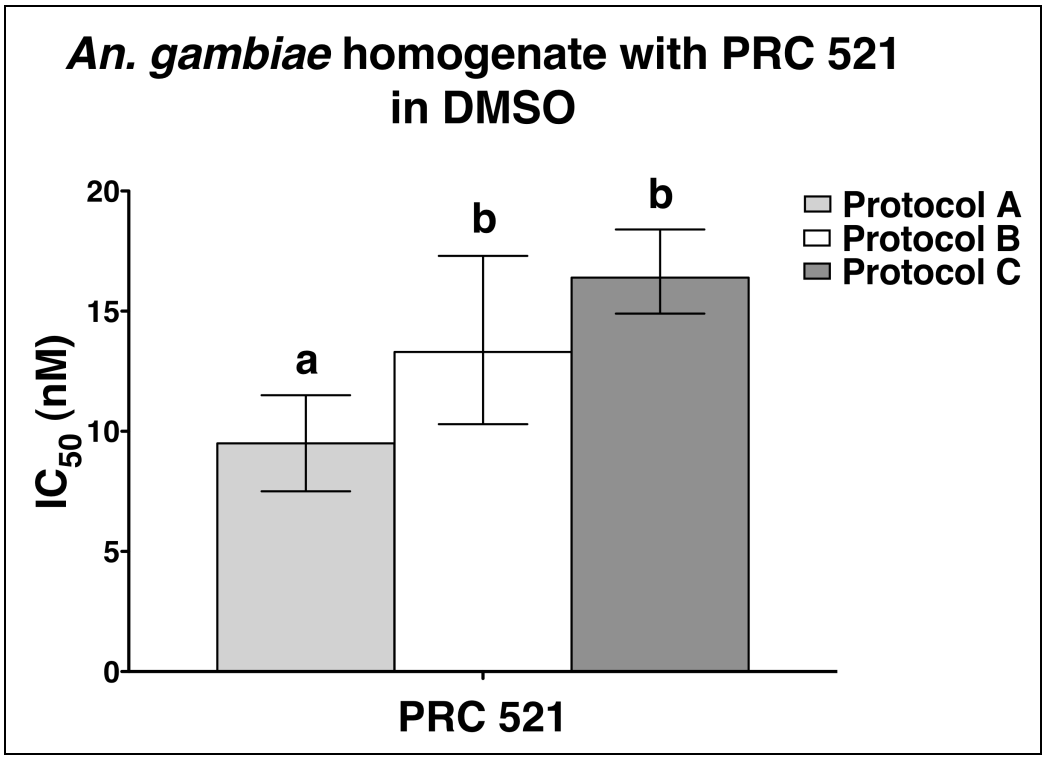

Figure 3.2. Comparison of three protocols on a non-selective experimental carbamate on *Ag*AChE exposed to DMSO. Columns represent IC_{50} values from non-linear regression analysis and were compared by a one way ANOVA and Tukeys multiple comparison test. Bars not labeled by the same letters represent statistical significance at $P < 0.05$. Error bars represent 95% CI.

Contrary to *Ag*AChE patterns of inhibition, *h*AChE yielded a maximum of 1.3-fold increase (PRC 337) in IC_{50} value with experimental carbamates or standard (non-selective) carbamates under the presence of 0.1 % DMSO (*v*/*v*) (Figure. 3.3). The decreased potency of experimental carbamates toward *Ag*AChE under the presence of 0.1% DMSO (*v*/*v*) and little to no statistical increase in IC_{50} value with *h*AChE produced drastically lower selectivity ratios than those previously found by Dr. Anderson (Table 3.2). The *h*AChE displayed little variance of IC_{50} values for Protocol A when EtOH was used as a solvent or when DMSO was used as a solvent (Table 3.3).

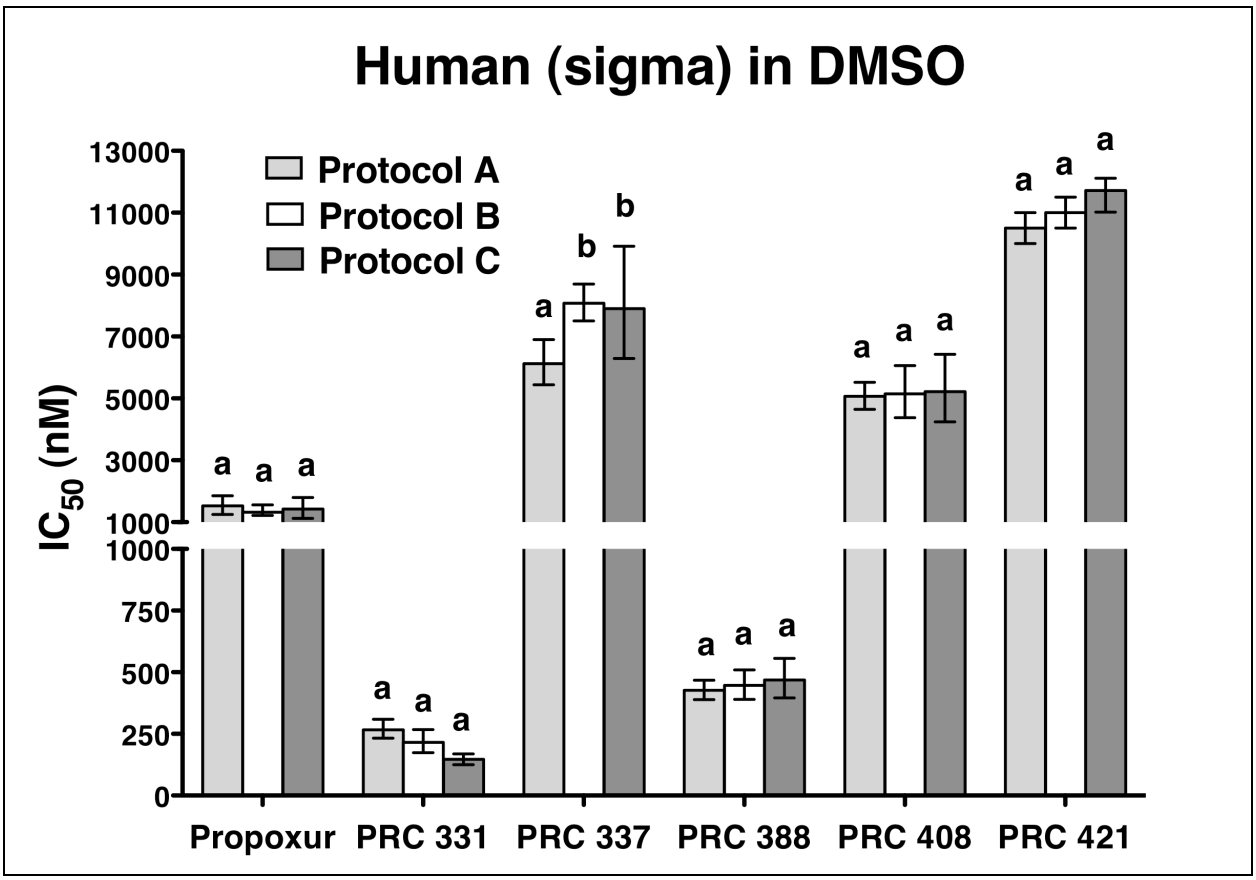

Figure 3.3. IC_{50} values for human recombinant enzyme exposed to DMSO. Columns represent IC_{50} values from non-linear regression analysis and were compared by a one way ANOVA and Tukeys multiple comparison test. Error bars represent 95% CI. Bars not labeled by the same letters represent statistical significance at $P < 0.05$. Note the change in Y-axis scale.

Table 3.2. Representation of reduced mosquito selectivity due to increased IC_{50} within *Ag*AChE under the presence of constant 0.1% DMSO (*v*/*v*), Protocol C.

	An. gambiae AChE 1[a]	Human AChE 1[a]		*An. gambiae* AChE 2[b]	Human AChE 2[b]	
Inhibitor	IC_{50}, nM; (95% CI)	IC_{50}, nM; (95% CI)	MS*	IC_{50}, nM; (95% CI)	IC_{50}, nM; (95% CI)	MS*
Propoxur	371 (320-421)	1710 (1420-2060)	5	527 (486-573)	1426 1128-1804	2.8
PRC 331	3 (2-4)	265 (240-293)	88	113 (106-120)	147 (115-197)	1.3
PRC 387	6 (3-9)	532 (375-756)	89	374 (306-450)	763 (522-991)	2.1
PRC 408	3 (2-4)	3627 (3182-4134)	1204	262 (249-277)	5219 (4242-6421)	19
PRC 421	276 (245-302)	98820 (89110-109600)	358	365 (350-377)	111719 (106100-116300)	306

*Mosquito Selectivity = IC_{50} of Human AChE / IC_{50} of mosquito AChE

[a] Data set 1 was taken from Dr. Troy Anderson at VA Tech Entomology (Anderson et al., 2008.) Inhibitors were prepared using Protocol A with EtOH as a solvent.

[b] Data set 2 was performed using Protocol C with constant 0.1% DMSO as a solvent.

Table 3.3. Comparison of human AChE inhibition with EtOH as a solvent and DMSO as a solvent.

	*h*AChE, Protocol A (EtOH)[a]			*h*AChE, Protocol A (DMSO)[b]		
Inhibitor	IC_{50} (nM; 95% CI)	Hill Slope	r^2	IC_{50} (nM; 95% CI)	Hill Slope	r^2
Propoxur	1710 1420-2060)	1.3	0.99	1524 (1250-1855)	1.05	0.99
PRC331	265 (240-293)	0.98	0.99	266 (216-327)	0.88	0.99
PRC 337	9551 (7695-11850	0.97	0.98	6126 (5437-6902)	1.1	0.99
PRC 408	3627 (3182-4131)	1.06	0.99	5064 (4649-5518)	1.05	0.99
PRC 421	98820 (89110-109600)	1.52	0.98	107,000 (96540-118600)	0.86	0.99

[a] Data set 1 was taken from Dr. Troy Anderson at VPI entomology (Anderson et al., 2008.)

[b] Data set 2 was prepared using Protocol A with DMSO as a solvent

D. melanogaster (*Dm*AChE) and *M. domestica* (*Md*AChE) displayed similar patterns of inhibition across the three protocols and were similar to *h*AChE (Figure 3.4). *Dm*AChE displayed no significant difference in inhibition across the three protocols with the standard carbamate propoxur or experimental carbamates PRC 331 and PRC 408. A 2.1-fold increase in IC_{50} value was seen for PRC 337 whereas a decrease in IC_{50} value was seen for PRC 388 (1.5-fold) and PRC 421 (1.3-fold), when 0.1% DMSO (*v/v)* is present (Figure 3.4). *Md*AChE also displayed no significant difference in inhibition across the three protocols with the standard carbamate propoxur or experimental carbamates PRC 337 and PRC 408. When compared to protocol A, protocol C yielded a modest decrease in IC_{50} values for experimental carbamates PRC 331 (1.2-fold), PRC 388 (1.9-fold), and PRC 421 (1.3).

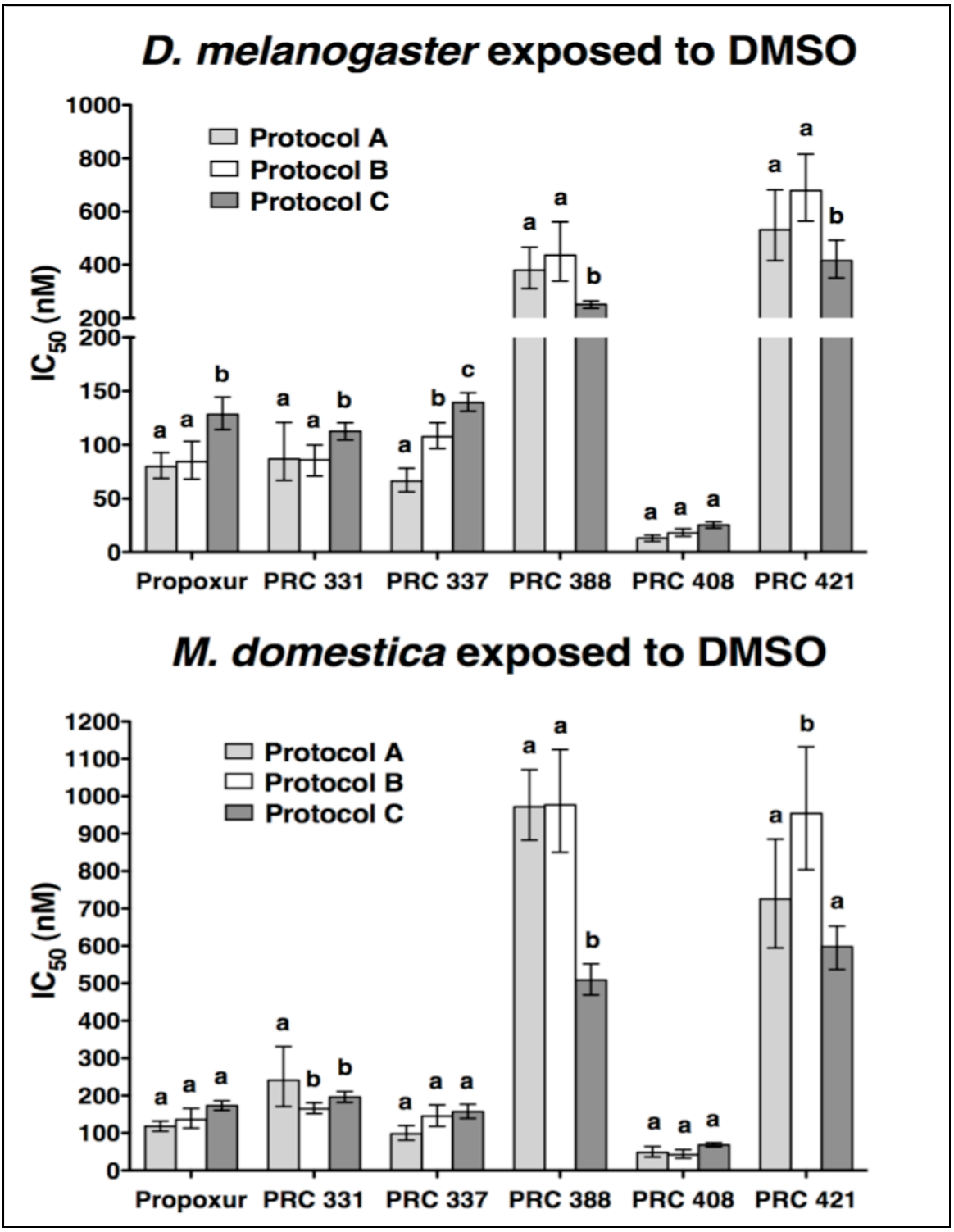

Figure 3.4. IC_{50} values for *D. melanogaster* and *M. domestica* AChE exposed to DMSO. Columns represent IC_{50} values from non-linear regression analysis and were compared by a one way ANOVA and Tukeys multiple comparison test. Bars not labeled by the same letters represent statistical significance at $P < 0.05$. Error bars represent 95% CI. Note the change in Y-axis scale.

EtOH effects toward Ag*AChE, and* Dm*AChE*

No statistically significant increase in IC_{50} values were seen across the three protocols among any species studied when EtOH was used as a solvent (Figures 3.5-3.7). EtOH had little effect on either *Ag*AChE enzymes when compared to results obtained when using DMSO. CBL and *Ag* homogenate displayed no statistical increase in IC_{50} values among the three protocols when EtOH is used as a solvent, regardless of the selectivity of the inhibitor (Figure 3.5). Similar IC_{50} values were observed for *Ag*AChE when using DMSO as solvent or EtOH as solvent. *h*AChE displayed no statistical increase in IC_{50} across the three protocols or the two carbamate classes when utilizing EtOH as the vehicle. Protocol C yielded statistically significantly lower IC_{50} values on experimental carbamates PRC 331 (2-fold decrease) and PRC 388 (1.5-fold decrease), when compared to Protocols A/B (Figure 3.6). Similar inhibition patterns were observed for *h*AChE with EtOH as a solvent (Figure 3.6) when compared to using DMSO as the solvent. The effects of EtOH on *Dm*AChE are shown in Figure 3.7 and were similar to those seen with DMSO as the solvent. No significant increase in inhibition was observed across the three protocols or the two classes of carbamates. Protocol C yielded a statistically significant lower IC_{50} value (3.5-fold decrease) in the presence of 0.1% EtOH (v/v) for PRC 331 (Figure 3.7).

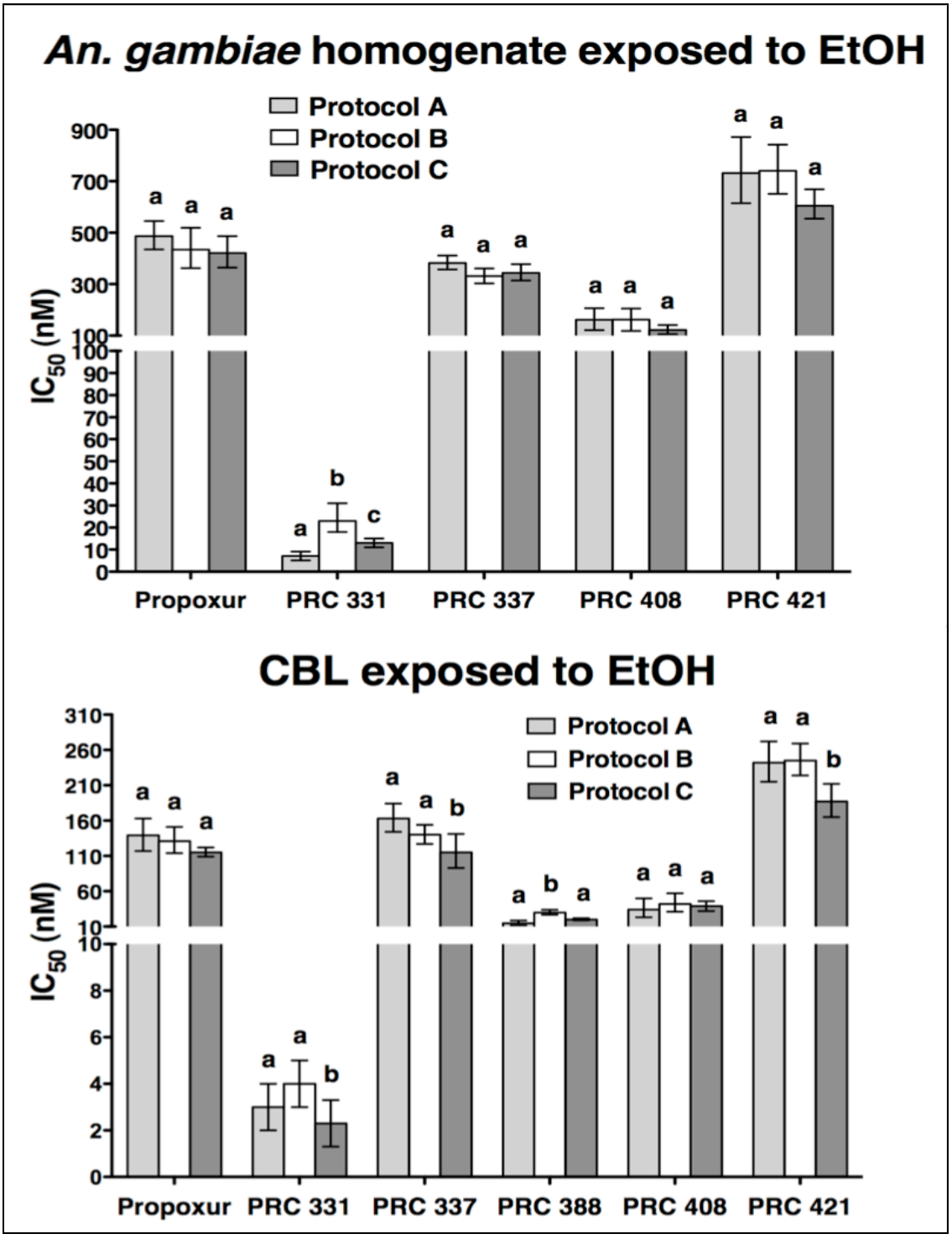

Figure 3.5. IC_{50} values for *An. gambiae* AChE exposed to EtOH. Columns represent IC_{50} values from non-linear regression analysis and were compared by a one way ANOVA and Tukeys multiple comparison test. Bars not labeled by the same letters represent statistical significance at $P < 0.05$. Error bars represent 95% CI. Note the change in Y-axis scale for CBL and *An. gambiae* homogenate.

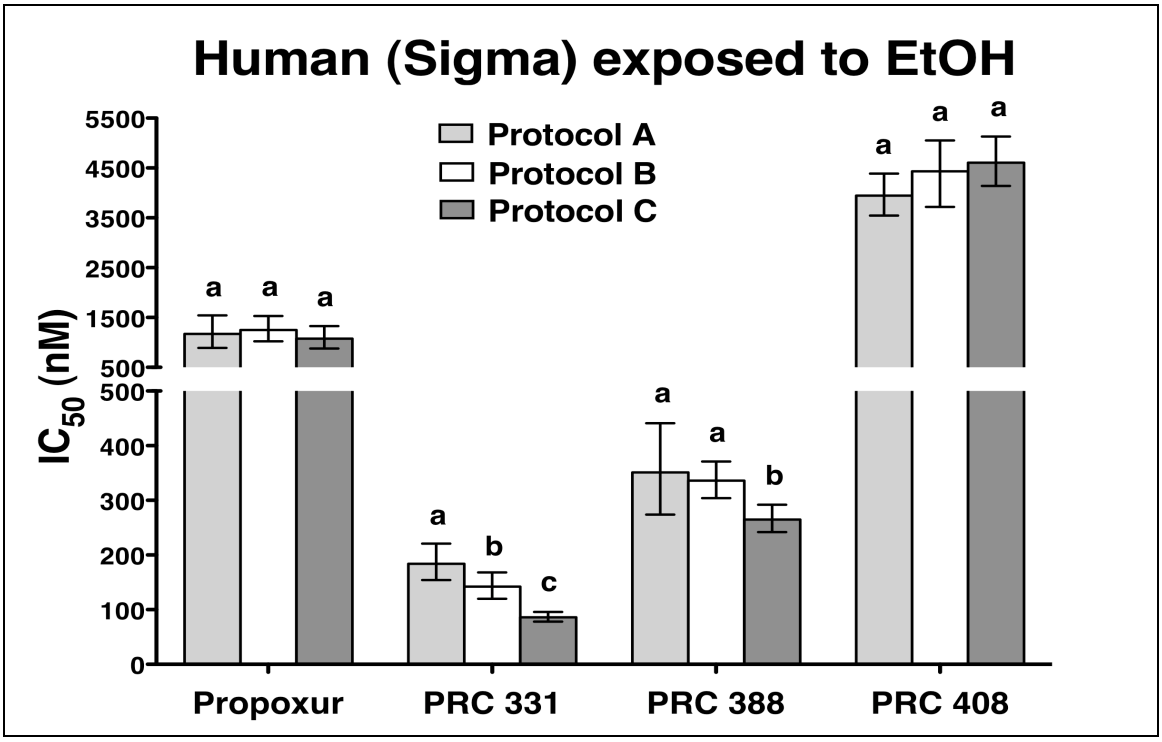

Figure 3.6. IC_{50} values for human recombinant AChE enzyme exposed to EtOH. Columns represent IC_{50} values from non-linear regression analysis and were compared by a one way ANOVA and Tukeys multiple comparison test. Bars not labeled by the same letters represent statistical significance at $P < 0.05$. Error bars represent 95% CI. Note the change in Y-axis scale.

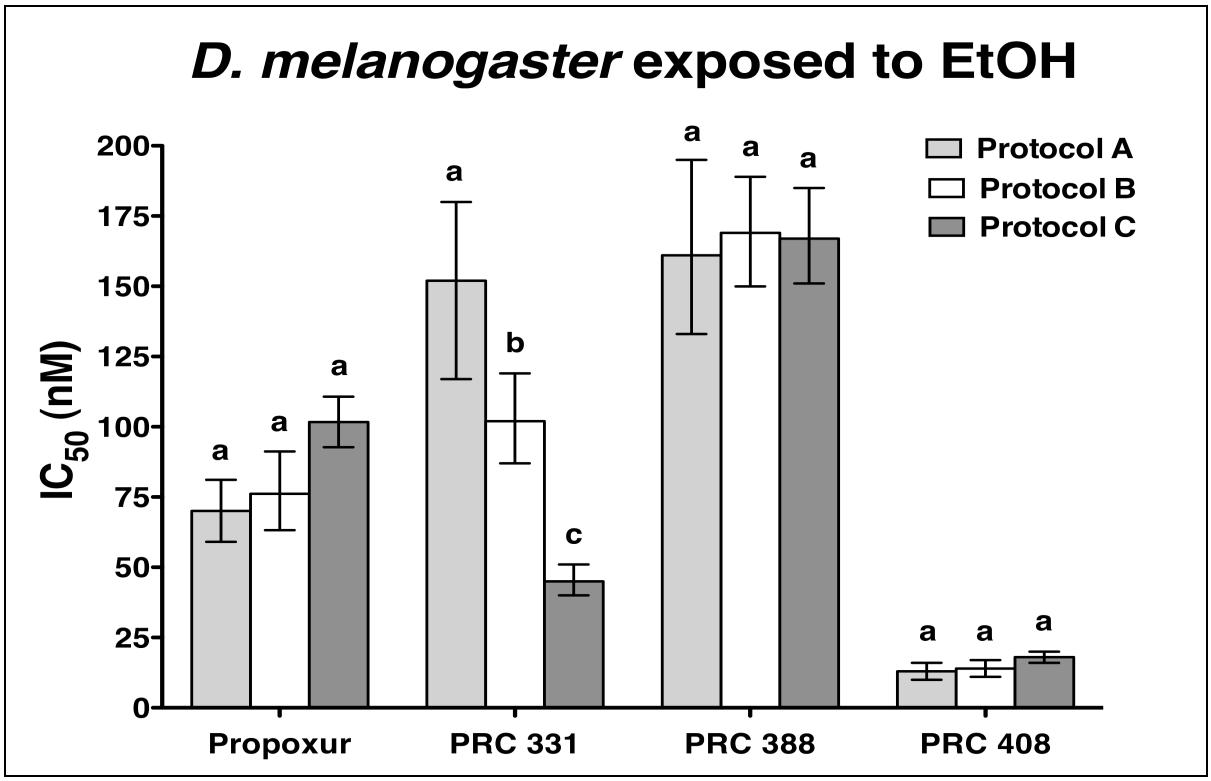

Figure 3.7. IC_{50} values for *D. melanogaster* AChE exposed to EtOH. Columns represent IC_{50} values from non-linear regression analysis and were compared by a one way ANOVA and Tukeys multiple comparison test. Bars not labeled by the same letters represent statistical significance at $P < 0.05$. Error bars represent 95% CI.

Influence of DMSO on AChE catalytic activity

To ensure that the decreased potency of the selective experimental carbamates was not an artifact of enzyme denaturation or DMSO-dependent AChE inhibition; we performed assays in which the inhibitor was replaced with comparable concentrations of DMSO, and results are shown in Figure 3.8. *Ag* homogenate showed 47% ± 2.5% inhibition at 10% DMSO, a 15% ± 0.85% increase in activity at 1% DMSO, and an increase of 1.1% ± 1.6% at the experimental concentrations of DMSO, 0.1% (*v*/*v*). CBL enzyme displayed 50% ± 4% inhibition at 10% DMSO, a 63% ± 3% increase in activity at 1% DMSO, and a 3% ± 1.7% increase in activity at 0.1% DMSO, the experimental concentration. *h*AChE displayed 96% ± 0.8% inhibition of

*h*AChE at 10% DMSO (*v*/*v*), 47% ± 5% inhibition at 1% DMSO (*v*/*v*), at the experimental concentrations of 0.1% DMSO (v/v), *h*AChE yielded a 7% ± 3% decrease in activity (Figure 3.8).

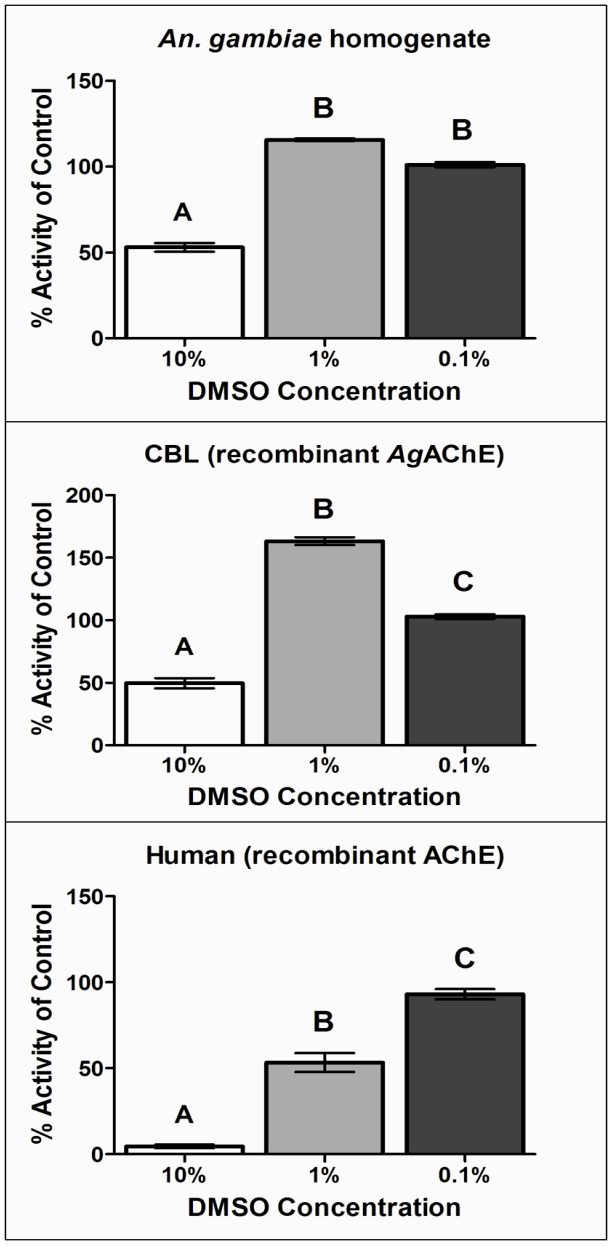

Figure 3.8. Influence of DMSO on AChE catalytic activity. Bars represent average (n = 3) percent activity remaining with error bars representing standard deviations. Bars not labeled by the same letters represent statistical significance at $P < 0.05$.

PRC 331 and *Ag* recombinant enzyme (CBL) were used to determine the midpoint of DMSO-dependent antagonism of inhibition, and is seen in Figure 3.9. The midpoint for DMSO effect on 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. The midpoint can also be noted as the EC_{50} for antagonistic effects of inhibition seen on *Ag*AChE enzyme. DMSO had substantial antagonistic effects on inhibition at 0.85 M (1% DMSO) to $10^{-3.85}$ M (10^{-3} % DMSO). We were unable to determine an EC_{50} for *Ag* homogenate, due to variability of response.

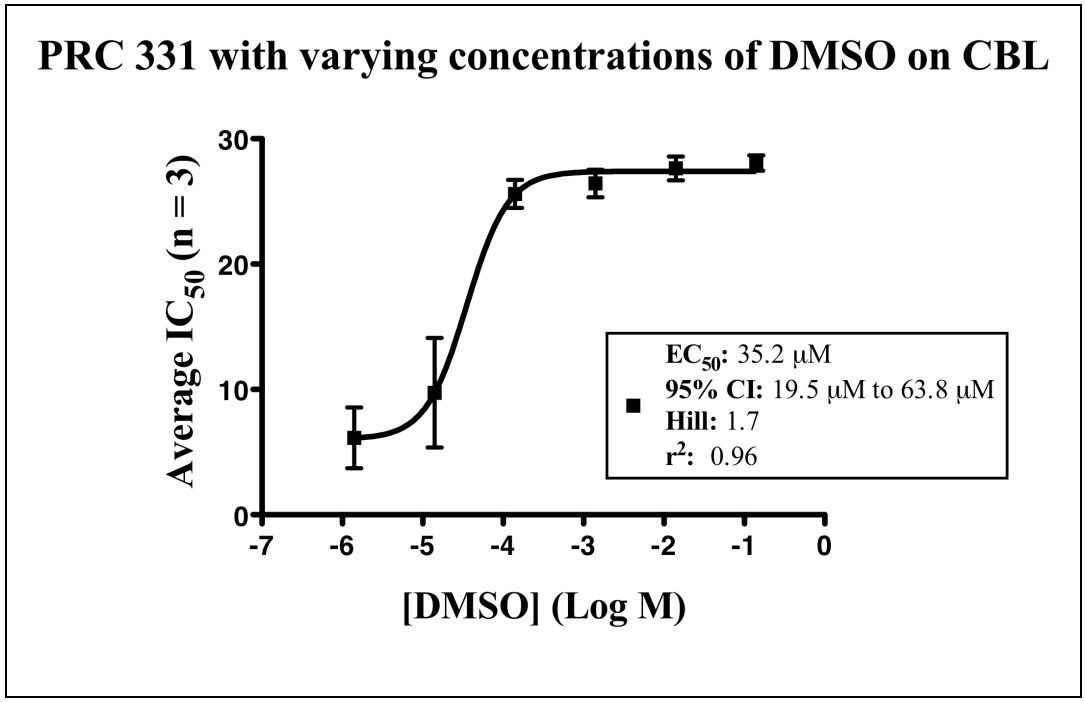

Figure 3.9. EC_{50} of DMSO effect on PRC331-dependent inhibition of recombinant *Ag*AChE (CBL).

DISCUSSION

Comparison of human AChE (*h*AChE) and *An. gambiae* AChE (*Ag*AChE) sequences portray numerous unique differences, such as Asp441/Tyr449 (*Ag*AChE/*h*AChE numbering), suggesting the potential development of selective anticholinesterases (Hosea et al., 1996). Figure 3.10 displays an overlay of *h*AChE and *Ag*AChE to visualize the Asp441/Tyr449 (*Ag*AChE/*h*AChE numbering) difference within a putative hydrophobic subpocket (Wong et *al*., unpublished). Work in our laboratory has identified multiple phenyl-substituted *N*-methylcarbamates which possess high selectivity of *Ag*AChE over *h*AChE (Table 1.1). These molecular models suggest alternate conformations of W84 and W431 (*Ag* numbering) within the hydrophobic subpocket of *Ag*AChE, giving the enzyme a high degree of flexibility. These alternate conformations of the corresponding residues in *h*AChE are not easily obtained due to the presence of Y449 (*h*) versus a D441 in *Ag*AChE. Crystal structures of *h*AChE reveal the presence of hydrogen bonds between Y449 and the indole nitrogens of W86 and W439 (human numbering), as shown in Figure 3.11. This interaction prevents flexibility within the hydrophobic subpocket of *h*AChE, rendering the gorge less accommodating to our branched alkane (at C-2 and C-3) carbamates. Within *Ag*AChE, Asp441 is analogous to Tyr449 of the human. Homology models of *Ag*AChE have suggested Asp441 is too distal to produce hydrogen bonds with W84 and W431, as seen in *h*AChE (Figure 3.12). The inability to hydrogen bond produces flexibility of W84/W431 within the hydrophobic subpocket of *Ag*AChE (Figure 3.12), potentially producing the high selectivity ratios of *Ag*AChE over *h*AChE.

The original selectivity ratios (Table 1.1) were determined using EtOH as a solvent in Protocol A. However, a decrease of inhibition on both *Ag*AChE enzymes was observed (Figure 3.1) upon rescreening of the experimental carbamates in the presence of a small amount DMSO

(0.1% [*v*/*v*]). This decrease was seen when DMSO was used as the solvent for Protocol C and compared to protocol A or B. However, there was no IC_{50} variance between DMSO or EtOH for *h*ACHE (Figures 3.3 and 3.6, respectively). The increase in *Ag*AChE IC_{50} values and stable *h*AChE IC_{50} values drastically reduces the selectivity ratios displayed in Table 1.1. This pseudo-reduction of selectivity due to solvent interactions has large implications for high-throughput screening of chemical libraries and will be discussed in subsequent sections.

This unexpected antagonism of inhibition by DMSO is potentially explained through the flexibility of W84 and W431 (*Ag* numbering). Models (Figure 3.13) suggest an interaction between a DMSO molecule and the indole nitrogens of W84 and W431 produces hydrogen bonds with Asp441 within *Ag*AChE (Wong et *al*., unpublished). Hydrogen bonding of these substituents produces an *Ag*AChE enzyme structure similar to that of *h*AChE and results in a loss of flexibility of the *Ag*AChE hydrophobic subpocket, producing a less accommodating enzyme for selective carbamates having bulky side chains. Thus, the stabilization of the *Ag*AChE enzyme through the presence of 0.1% DMSO (*v*/*v*) is potentially responsible for the solvent-dependent antagonism of inhibition seen with our *Ag*AChE selective carbamates. Thus, it is concluded that the inhibition of antagonism is due to this stabilization (allosteric), and not to direct competition with the insecticide for its binding site.

This antagonism was only observed when using DMSO as the solvent. Identical studies were performed in the presence of 0.1% ethanol (EtOH) (*v*/*v*), but no antagonistic effects on inhibition were observed. There are a number of molecular differences between DMSO and EtOH that could potentially explain why we observed no increase in IC_{50} value under the presence of 0.1% EtOH (*v*/*v*). A principal difference between the two solvents is the molecular size. EtOH has a polar surface area of 9.23 $Å^2$ and a molar volume of 59 cm^3 (ChemSpider – 1),

whereas DMSO is a larger molecule with a polar surface area of 36.28 $Å^2$ and a molar volume of 71 cm^3 (ChemSpider – 2). Therefore, it is probable to note that, due to its smaller size, EtOH is not capable of forming hydrogen bonds between W84/W431 and Asp441.

The antagonistic effect of DMSO is also directly linked with selectivity of the carbamates. Two standard carbamates (propoxur and bendiocarb) were tested on both *Ag*AChE enzymes and resulted in little to no statistical difference for inhibition among the three protocols. However, there are obvious antagonistic effects with the selective carbamates on *Ag*AChE in the presence of 0.1% DMSO (*v/v*) (Fig. 3.1). The experimental carbamates are highly branched at the C-2 and C-3 positions. A three way branched *(tert* butyl) silane at the C-3 position (PRC387) alkane or a two way branched alkane (with the branches being longer than isopropoxy) at C-2 likely allow interactions (flipping of W84) to allow for specificity. No DMSO-dependent antagonism of inhibition was observed with the non-selective carbamates, potentially due to alternate branching schemes (when compared to the experimental carbamates) at C-2 and C-3 or poor flexibility of the compound. Bendiocarb is a somewhat larger compound that, due to its structure, yields little flexibility in the leaving group, and therefore prevents appropriate interaction with W84, producing poor specificity. The structural conformations at C-2 and C-3 apparently allow the standard carbamates to react with the catalytic acyl site by bypassing the hydrogen-bonded hydrophobic subpocket within *h*AChE and the DMSO-stabilized hydrophobic subpocket of *Ag*AChE. Thus, the presence of DMSO has no effect on enzyme inhibition when commercial non-selective carbamates are used. A non-selective experimental carbamate (PRC 521, 3-*sec*-butyl) was also tested to ensure decreased inhibition is not an artifact of our experimental insecticides. This also resulted in little (1.7-fold increase from Protocols A to C)

statistical difference between the three protocols indicating that the structural specificity on inhibition is quite precise (Fig. 3.2).

Along with the solvent used and the selectivity of the inhibitor, the species is a factor in the solvent-dependent antagonism of inhibition. Two primary genes have been discovered which encode AChE, *ace-1* and *ace-2* (Weill et al., 2004). Previous studies have shown that AChE1 is the primary site for organophosphate and carbamate binding, implying that the *ace-1* gene encodes the primary AChE in many insect species (Chen et al., 2009). Therefore, it is believed that the insect *ace-1* gene is generally more important in comparison with 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. Two *ace* genes, *ace-1* and *ace-2*, were found to encode *Ag*AChE (and other mosquitoes) however; it is well established that *ace-1* is the primary gene for encoding the AChE enzyme within *Ag*AChE (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).

In this study, *Drosophila melanogaster* AChE (*Dm*AChE), *Musca domestica* AChE (*Md*AChE), and *h*AChE were tested to determine any solvent-dependent antagonism of inhibition with DMSO on species other than *Ag*AChE. All species had little to no increase in IC_{50} values among the three protocols when either DMSO or EtOH was used. However, it is interesting to note that PRC 388 and PRC 421 had significantly lower IC_{50} values and therefore greater potency for both *Dm*AChE and *Md*AChE enzymes when small amounts of DMSO were present *(*Figure 3.4). Presumably, the decrease in IC_{50} value is due to variance in amino acid

sequences and/or structure of the AChE gorge. Sequence alignment of *Dm*AChE and *Ag*AChE displayed very similar sequences within the hydrophobic subpocket (W84/W83, W431/472, and D441/D482) (*Ag*/*Dm* numbering) (Harel et al., 2000). Although *Dm*AChE has a similar sequence within the hydrophobic subpocket, other differences in the sequence may prevent DMSO from binding to D482 in *Dm*AChE and form hydrogen bonds with W83 and W472 (*Dm* numbering). For example, *Ag* has a M438 (*Ag* numbering) near the hydrophobic subpocket, while *Dm*AChE possesses a L479 (*Dm* numbering). This, or other differences, could potentially affect the interaction between DMSO and the enzyme.

Substantial work has been performed on DMSO and its effect toward enzymes, decreased potency due to freeze thaw methods, and the effect of water on DMSO (Cheng et al. 2003; Kozikowski et al., 2003). There has also been documentation of DMSO acting as a denaturant (Bhattacharjya and Balaram, 1997; Jacobson and Turner, 1980; Fujita et al., 1982; Kovrigin and Potekhin, 1997) and/or an inhibitor (Perlman and Wolff, 1968; Kleifield et al., 2000; Johannesson et al., 1997) toward enzymes. Studies have also shown enzyme activation under the presence of DMSO (Rammler, 1963), similar to the increase in *Ag*AChE catalytic activity at 1% DMSO, shown in Figure 3.8. However, there has been little documentation of any effect that DMSO might have on the pharmacological actions of compounds. Knowledge of interactions between solvent and proteins/ligands is vital for valid high throughput screening and development of selective inhibitors.

Within HTS screens, DMSO concentrations usually range from 0.1% (*v*/*v*) to 5% (*v*/*v*), depending on the required inhibitor concentration range, assay type, etc. (Tjernberg et al., 2006). Many researchers perform *in vitro* assays or HTS screens at solvent concentrations that simply do not affect the enzyme through denaturation or inhibition. However, these amounts of DMSO

may be problematic for some screens, as we demonstrated a solvent-dependent antagonism of inhibition at levels (0.1% DMSO (*v*/v)), which had little to no direct effect on the maximal catalytic activity of the enzyme (Figure 3.8). We found the midpoint (EC_{50}) of DMSO effect toward *Ag*AChE enzyme inhibition to be 35.2 μM. Assays performed at 0.1% DMSO (*v/v*) contain 14.08 mM DMSO. This concentration, as shown from my work, has a high probability of affecting the results of HTS screens.

Due to the fact DMSO has been used as the standard solvent for HTS screens (Tjernberg et al., 2006), the reduction of selectivity herein described or other similar effects are potentially capable of occurring within the industrial HTS screening process. This spurious appearance of low selectivity via DMSO effects might remove selective and promising insecticides from commercial consideration, thus preventing further testing and potential marketing of the compound. Such an effect of DMSO might also impact other protein/ligand systems, including pharmaceuticals.

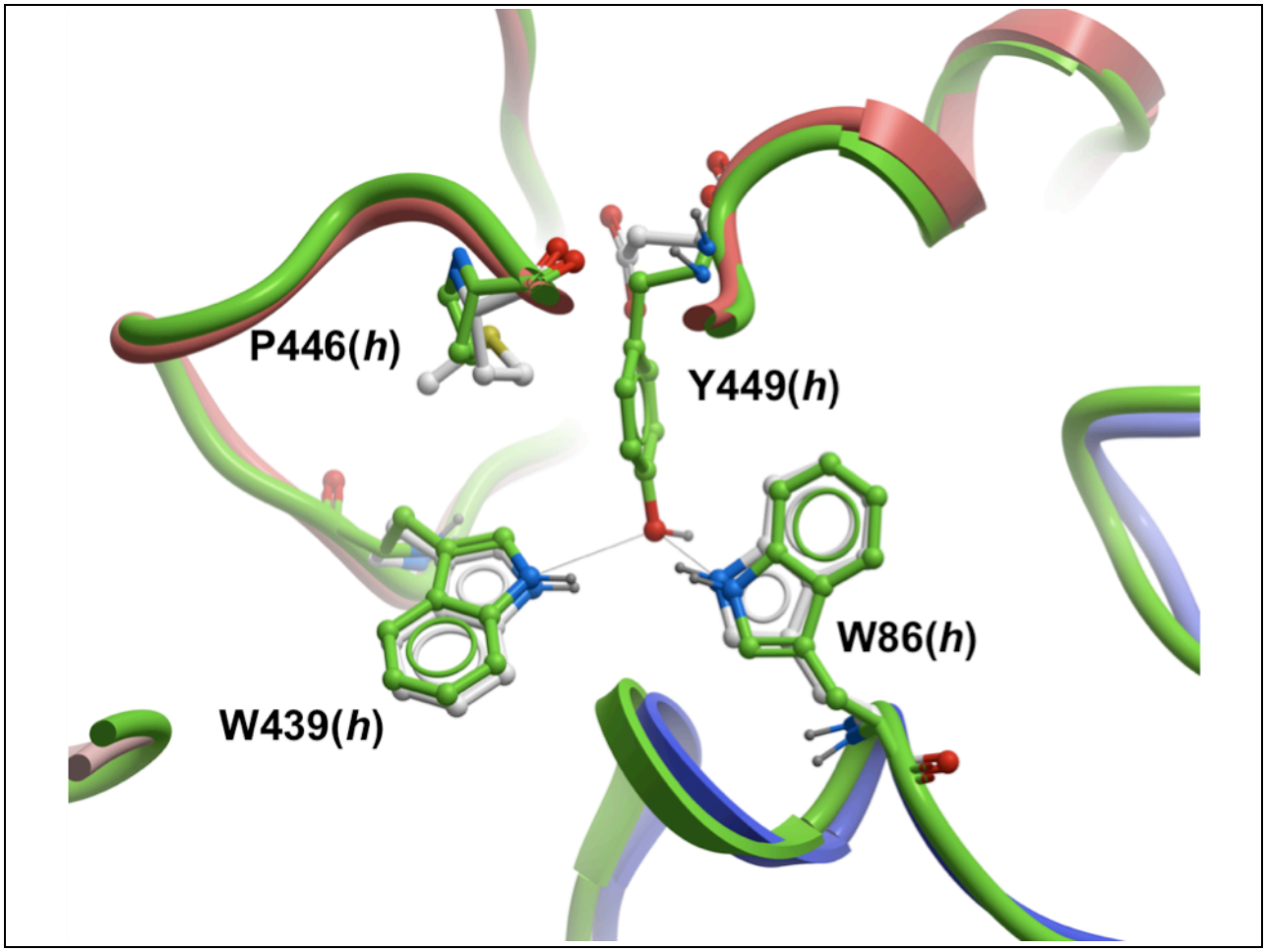

Figure 3.10. Overlay of *h*AChE (Fig. 3.11) and *Ag*AChE (Fig. 3.12). The model was created by Dr. Dawn Wong (VA Tech Entomology) using ICM Browser Pro version 3.7 (Molsoft LLC, La Jolla, CA, USA). Grey dashes represent hydrogen bonding between Y449 (*h)* and W86/W439 (*h*). Green ribbons represent *h*AChE. Blue and red ribbons represent *Ag*AChE and orient from N- to C- terminus respectively. Permission to use granted by: Drs. Totrov and Wong.

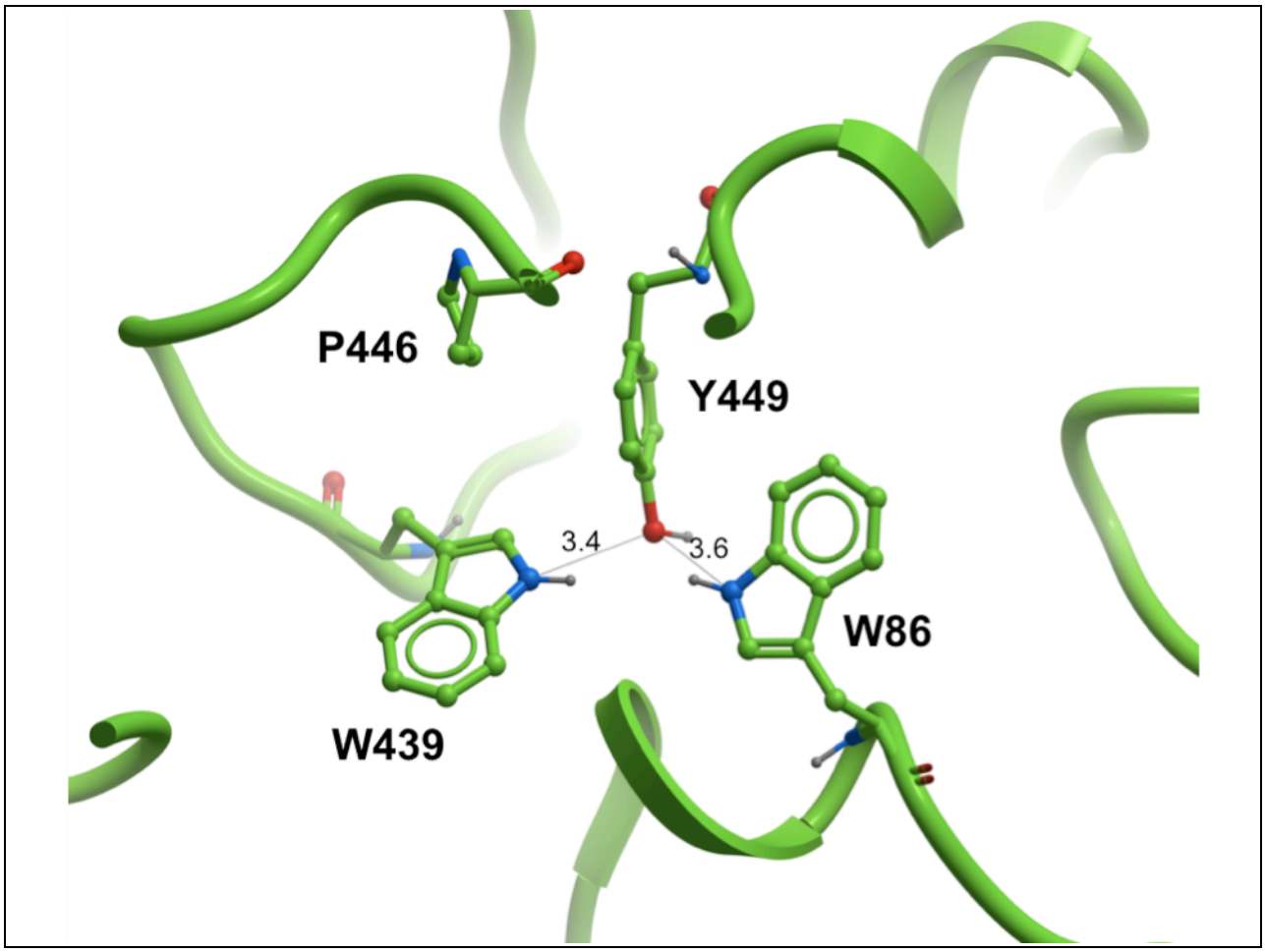

Figure 3.11. X-ray crystal structure of *h*AChE alone (green ribbons, PDB ID 1b41). Significant residues found within the hydrophobic subpocket are shown as ball and stick model, with green carbon atoms. Grey dashes represent hydrogen bonding between Y449 (*h)* and W86/W439 (*h*) (interatomic distances in Å). Model was created by Dr. Dawn Wong (VA Tech Entomology) using ICM Browser Pro version 3.7 (Molsoft LLC, La Jolla, CA, USA). Permission to use granted by: Drs. Totrov and Wong.

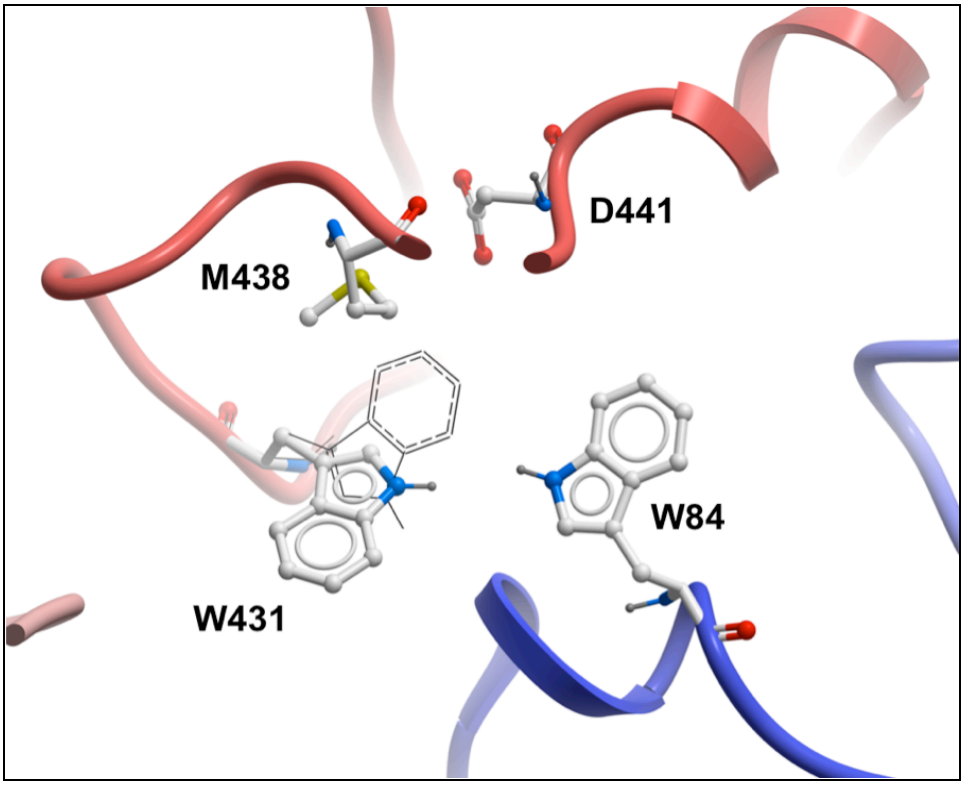

Figure 3.12. Monte Carlo-refined *Ag*AChE homology model. It displays selected residues found within *Ag*AChE hydrophobic subpocket and are shown as CPK-colored ball and stick model, with white carbon atoms. Alternate conformation of W431 (*Ag)* is caused by the binding of a selective ligand and is displayed as black lines. Blue to red ribbon represents N- to C- terminus. Model was created by Dr. Dawn Wong (VA Tech Entomology) using ICM Browser Pro version 3.7 (Molsoft LLC, La Jolla, CA, USA). Permission to use granted by: Drs. Totrov and Wong.

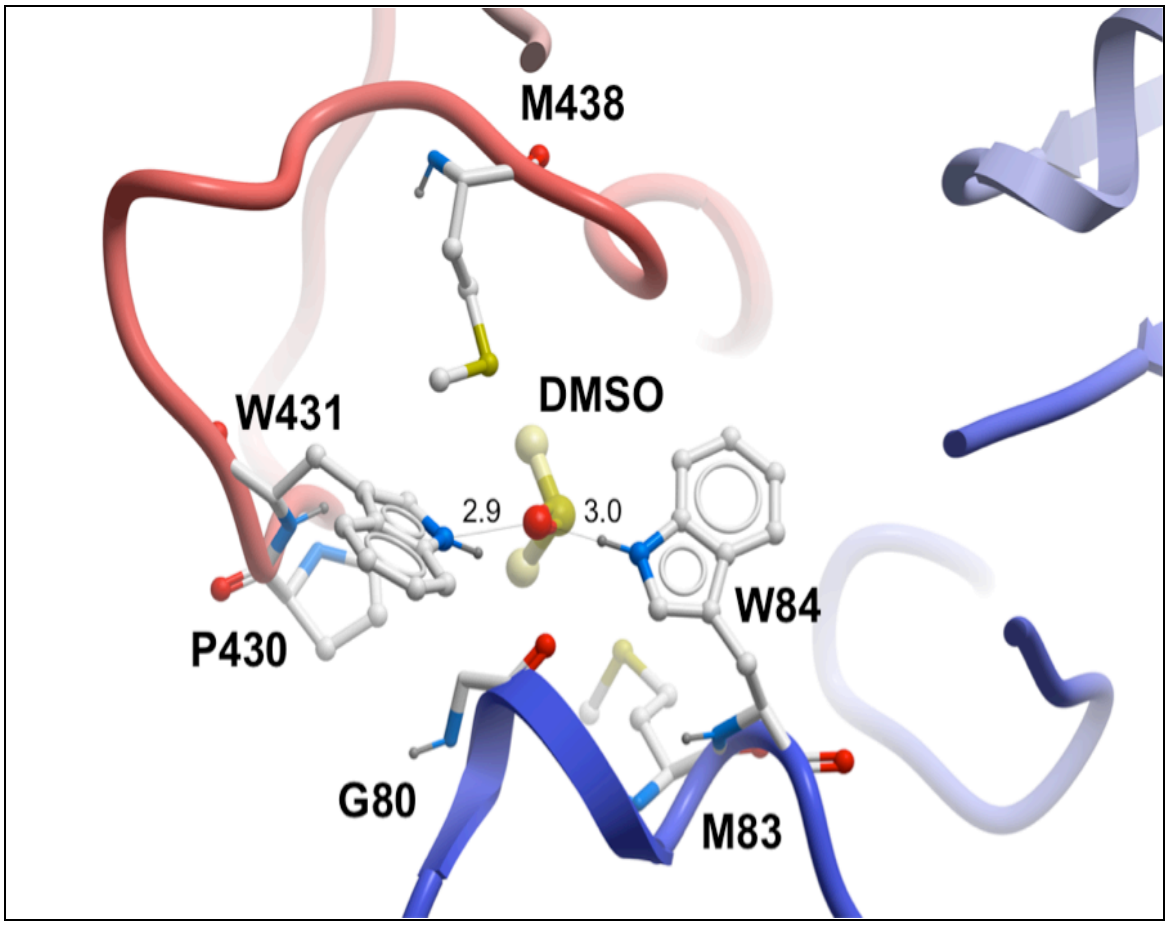

Figure 3.13. Top view of *Ag*AChE with DMSO molecule bound at the 'allosteric' hydrophobic subpocket. Bridged hydrogen bonding is shown between the DMSO oxygen and W84 (*Ag*)/W431 (*Ag)* with interatomic distances in Å. Bound DMSO molecule, rendered as ball and stick model with pale yellow carbon atoms, is closely flanked by two methionines, M83(*Ag*) and M438(*Ag*), and several other side chains. Model was created by Dr. Dawn Wong (VA Tech Entomology) using ICM Browser Pro version 3.7 (Molsoft LLC, La Jolla, CA, USA). Permission to use granted by: Drs. Totrov and Wong.

Chapter 4

CONCLUSION

The use of insecticides for mosquito control remains the most important and effective component of the integrated vector management approach for the control of vector borne diseases (Hemingway and Ranson, 2000). However, increased prevalence of resistance among vectors combined with the ongoing banning of insecticides is forcing researchers to develop novel insecticides that possess high selectivity ratios of mosquito over human enzyme.

Work in our laboratory has identified several species-selective carbamate insecticides that possess high selectivity ratios of *Ag*AChE vs. *h*AChE. Selectivity ratios were determined using protocol A and EtOH as the solvent. However, upon rescreening of selective *Ag*AChE inhibitors with a small percentage (0.1% (*v/v*)) of DMSO present, we observed statistically significant antagonistic effects toward inhibition. IC_{50} values increased up to 43-fold for *Ag* homogenate and 28-fold for CBL. Non-selective inhibitors (propoxur, bendiocarb, and PRC 521) displayed little to no DMSO antagonistic effects toward inhibition on either *Ag*AChE enzyme. Contrary to the effect of DMSO on *Ag*AChE, the other enzymes studied, *h*AChE, *Dm*AChE, and *Dm*AChE, displayed no significant antagonism of inhibition in the presence of DMSO. To determine if this effect was due to DMSO itself or it was an artifact of any organic solvent, we performed identical experiments under the presence of EtOH. We saw no antagonistic effects with ethanol among any species studied, including the *Ag*AChE enzymes. Therefore, we concluded this interaction is species-dependent, solvent-dependent, and inhibitor-dependent.

Molecular models produced by Drs. Wong and Totrov suggest the selectivity of our novel carbamates is due to flexibility of W84 and W431 (*Ag* numbering) within the hydrophobic subpocket of *Ag*AChE. The corresponding residues within *h*AChE are hydrogen bonded with

Y449 (*h),* producing a rigid enzyme structure within the hydrophobic subpocket. This flexibility of W84 (*Ag*) and W431 (*Ag*) may also be responsible for the solvent-dependent antagonism of inhibition observed in this research. Molecular models suggest that DMSO is capable of hydrogen bonding with the indole nitrogens of W84/W431 (*Ag* numbering) and D441 within *Ag*AChE, forming a DMSO stabilized 'allosteric' subpocket. This DMSO stabilization causes *Ag*AChE to have a rigid structure, similar to *h*AChE, inhibiting the highly branched (selective) experimental carbamates from binding to the target site.

This species-, solvent-, and inhibitor-dependent phenomenon has large implications for the high throughput screening process, as usually performed by industrial chemical companies. The midpoint for DMSO-dependent antagonism was found to be 35.2 μM, or 0.00025 % DMSO. Many pharmaceutical companies screen their chemical libraries at DMSO concentrations ranging from 0.1% (*v/v*) to 5% (*v/v*) (Tjernberg et al., 2006). This is substantially higher than DMSO midpoint of antagonism, indicating potential discrepancies within results of pharmaceutical companies.

Companies that continue to use one solvent and one protocol to screen their chemical library have a high probability of overlooking selective and potent insecticides. Accounting for interactions between the solvent and protein/ligand is vital for the development of appropriate HTS methods and must be studied prior to screening of chemical libraries. Similar types of solvent interactions may impact data on potential pharmaceuticals.

Future Studies

In this work, *An. gambiae* AChE was found to display an increased IC_{50} value in the presence of 0.1% DMSO (*v/v*). Although other insect species were studied and exhibited no antagonistic effects toward inhibition, other mosquito vectors such as *Culex quinquefasciatus*

and *Aedes aegypti* should be studied to determine if this effect is specific to *Ag*AChE or if increased IC_{50} values are also observed among other mosquito species under the presence of 0.1% DMSO (*v/v*). This can have large implications toward insecticide design for control of dengue fever, lymphatic Filariasis, west Nile virus, and many other mosquito vectored diseases.

Secondly, it is important to confirm whether binding of DMSO within AChE is competitive or non-competitive with respect to the ligand (*i.e.* inhibitor). By determining the binding kinetics of DMSO, advances can be made in the design of selective insecticides through an understanding of the interactions between DMSO, ligands, and the enzyme.

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