Evaluation of Novel Carbamate Insecticides for Neurotoxicity to Non-Target Species

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Abstract

Malaria (vector: *Anopheles gambiae*) is a major infectious disease that kills about 1 million people each year. For the improvement of its treatment and vector control during the past decades, several issues such as high medicine cost, insecticide resistance, and lack of an effective vaccine have prevented adequate control of malaria. Additionally, the low selectivity of malaria vector insecticides also presents a public health problem. The purpose of developing novel carbamate insecticides in our laboratory is to offer effective and selective insecticide options to achieve the ultimate goal of malaria control.

First, 50% inhibition concentration (IC$_{50}$) data was collected from three mammalian AChEs with eight commercial carbamate insecticides by using the Ellman assay. The IC$_{50}$ values varied from 57 nM to 7358 nM. The AChE sensitivity pattern and level were shown to be similar between the recombinant mouse and ICR male mouse brain cortex homogenate (slope = 0.99, R$^2$ = 0.96). Then eight novel carbamate insecticides that are possible malaria vector control agents were selected for further neurotoxicity testing in non-target organisms. For commercial carbamate insecticides, the IC$_{50}$ varied from 9.1 nM to 2,094 nM. For the novel carbamate insecticides, it varied from 58 nM to 388,800 nM. Based on IC$_{50}$ data from previous work on *A. gambiae*, the selectivity index (IC$_{50}$ of non-target species / IC$_{50}$ A. gambiae) ranged from 0.17 to 5.64 and from 0.47 to 19,587 for commercial and novel carbamate insecticides, respectively.

Subsequently, the AChE protein sequence alignment comparison and cladogram were used to compare the genetic and evolutionary relationship among five different organisms. The alignment
score ranged from 88 for mouse vs. human to 54 for hen vs. T. californica. The evolutionary relationships among species was obtained from the cladogram. Recombinant mouse vs. recombinant human was shown to have the most similar inhibitor potency profiles (alignment score = 88, closest taxa position on cladogram, similar AChE sensitivity pattern \( R^2 = 0.81 \) and level \( P > 0.05 \) to the novel carbamates).

Neurotoxic esterase (NTE) assay showed that the novel carbamates did not significantly inhibit NTE, inhibition of which underlies a significant hazard for anticholinesterases, especially organophosphates, in several nontarget vertebrate organisms. The NTE activity in the presence of novel carbamate insecticides ranged from 93% to 116% of the control, while in the commercial group, bendiocarb significantly inhibited NTE, leaving only 76.5% of the initial reactivity at 1 mM inhibitor concentration.

Further in vivo bioassay using Daphnia magna was conducted to compare the aquatic toxicity of commercial and novel carbamates. The data showed that except for PRC331 (3-tert-butylphenylmethylcarbamate), all novel carbasates were of similar potency as bendiocarb (LC\(_{50} = 611\) nM) for aquatic toxicity, and their LC\(_{50}\) values ranged from 172 nM (PRC331) to 1109 nM.

In conclusion, the novel carbamate insecticides would appear to be an improvement over commercial carbamate insecticides because of greater selectivity, negligible NTE inhibition capacity, but in some cases with potent in vivo toxicity to Daphnia magna. However, since the envisioned usage of these compounds is in bednets or as indoor residual sprays (IRS), any environmental exposures to nontarget aquatic organisms are expected to be minimal.
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Chapter 1: Introduction and Review of Literatures

1.1. General Introduction of Malaria and Current Malaria Control Methods

Malaria, which is a vector-borne disease, remains an urgent global health issue and once again has the attention of the international health community (Na-Bangchang and Congpuong 2007; O’Meara et al. 2010; Ringwald 2007). According to data from the United Nations Children’s Fund (UNICEF) in 2008, malaria is still a widespread disease, particularly in many countries of Africa and Asia, which infects approximately 100-300 million people and kills 1-3 million people every year (Anonymous 2008). In addition, in Africa, more than 2500 children under 5 years old are killed every day because of malaria (Ringwald 2001). So there is an urgent need for malaria control.

Current malaria control methods include epidemiological prevention and chemotherapy. Although searching for new medicines for malaria chemotherapy for many years, the clinics so far are still heavily dependant on limited compounds. These compounds belong to four categories, including the 4- or 8-aminoquinoline group, the antifolate group, the artemisinin group and the hydroxynapthoquinone/atovaquone group (Croft et al. 2002; Popov et al. 2003; Schlitzer 2008). Because of limited chemical compound diversity (Jamshidzadeh et al. 2007), more resistant malarial pathogen infection cases and untreatable cases have been reported (Dondorp et al. 2009; Na-Bangchang and Karbwang 2009; Schlagenhauf and Petersen 2009). In addition, lack of an efficient malaria pathogen vaccine makes disease control even more difficult (Tetteh and Polley 2007; WHO 2005). As a vector-borne disease, the transmission of malaria depends highly on its insect vector, which are mosquitoes of the genus Anopheles, including Anopheles gambiae (Beier et al. 1990). In 2007, WHO reconfirmed the importance of malaria vector control in malaria reduction programs (Van den Berg 2009). In Africa, it has been reported that after appropriate insecticide usage, there is
a significant reduction in the parasite prevalence and malaria diagnosis (Bhattarai et al. 2007). On top of that, compared to antimalarial medicines, the relatively high cost-efficiency of insecticides makes them more affordable for countries suffering from malaria (Conteh et al. 2004). In conclusion, the growing emphasis on malarial vector control impels the attention on insecticide research.

Carbamate insecticides belong to an insecticide category that targets acetylcholinesterase (AChE). Carbamates have been widely applied onto bednets and as indoor sprays in malarial control in recent years and achieved some positive results (Akogbéto et al. 2010; Djènontin et al. 2009; Djènontin et al. 2010), but the increasing number of reports finding carbamate- and organophosphate-resistant *A. gambiae* stains requires the discovery of alternative anticholinesterases (Boon et al. 2008; Corbel et al. 2003; Mostafa and Allam 2001; N'Guessan et al. 2003). In the following pages, brief introduction will be given on the structure, pharmacodynamics, pharmacokinetics, and toxicity of carbamate and organophosphate insecticides. Furthermore, promising data and structures of newly developed novel carbamate insecticides from Dr. Jeffrey Bloomquist’s and Dr. Paul Carlier’s collaboration will be discussed.

### 1.2. Carbamate Compounds

A carbamate compound active on acetylcholinesterase was first discovered in the extract of *Physostigma venenosum* (calabar bean) (Proudfoot 2006). The first described carbamate anticholinesterase was called physostigmine (Figure 1b), which is an ester form of methylcarbamate. As an anticholinesterase, physostigmine was used initially as one treatment option for myasthenia gravis (Flacke 1973). In addition, the symptoms of glaucoma, Alzheimer's disease, and delayed gastric emptying can also be relieved through the same mechanism (Rathmann and Conner 2007,
Loewenstein et al. 1993, Ulvestad and Gerner 1984). In this thesis, I will focus on the discussion of carbamates used for insecticidal purposes.

1.2.1. Structure of Carbamates

Generally speaking, carbamate compounds are colorless, odorless, crystalline powders at room temperature (Baron 1991), and are derived from their parent compound, carbamic acid, whose generic structure is NH₂COOH. The structures of example carbamate compounds are shown in Figure 1-1. The functional group in carbamate compounds is also called urethane and defined as R₁R₂NCOOR₃, where R₁ and R₂ represent chemical groups including hydrogen and short-chain alkyls, such as methyl and ethyl groups, while R₃ is usually oxime or cyclic unsaturated hydrocarbon such as phenol or naphthol. The substituent at R₁ and R₂ positions will change the binding of carbamate compounds with AChE greatly, which is the basis for selectivity and structure-activity relationships of carbamate insecticides.

Figure 1-1. Structure of carbamate compounds. a. General structure of carbamate compounds. R₁ and R₂ represent chemical groups including hydrogen and short-chain alkyl. R₃ is usually a cyclic unsaturated hydrocarbon. b. Structure
absorption, distribution, metabolism and excretion (Timchalk 2006).

1.2.2. General Metabolism of Carbamates

The pharmacokinetics of carbamate compound in the animal body includes 4 steps, which are absorption, distribution, metabolism and excretion (Timchalk 2006).

1.2.2.1. Absorption of Carbamates

Three pathways for insecticide absorption have been demonstrated, including oral exposure (Bardin et al. 1994), dermal exposure (Johansson 1992), and respiratory exposure (Baban et al. 1998). Oral exposure is thought to be the most important route for carbamate absorption. Exposure usually happens during food intake because of carbamate insecticide residuals on food (Barnat et al. 2010; Boon et al. 2008). Other examples can occur, such as intentional ingestion for suicide purpose (Ameno et al. 2001; Barnat et al. 2010). Likewise, dermal exposure is also an important exposure route, which is more prevalent in certain groups of people. It has been reported that workers involved in agricultural product processing, such as tobacco (Lonsway et al. 1997), have much higher carbamate compound deposition in their body than the average population (Calli and Marinovich 1987). This deposition is thought to mainly come from the dermal pathway. In addition, during carbamate insecticide production and preparation, such as mixing and loading, workers can also be exposed through dermal contact (Calli and Marinovich 1987). Although not considered as a major route, carbamate compound exposure through inhalation is still considered a possible route because some carbamate compounds are volatile (Hall and Harris 1979). Additionally, the airway is also an important exposure route during agriculture production, where workers inhale carbamate spray drift or contaminated dust during insecticide application (Hall and Harris 1979). Large doses
of carbamate through inhalation could cause pulmonary edema and respiratory stress, which is thought to be a major reason of death by carbamate intoxication (Salisbury et al. 1974).

### 1.2.2.2. Distribution of Carbamates

Upon absorption, carbamate compounds distribute throughout the body rapidly. Ahdaha et al. (1981) found that the half-life is from 8 to 17 minutes for carbamate penetration through organs. Pharmacokinetic research further suggested that fast distribution of carbamate compounds fits a one-compartmental model (Timchalk 2006). For example, one large oral dose administrated in the human body, of carbamate (chlorphenesin) reached serum concentrations, with mean lag times and half-lives for absorption of a one compartmental model (Forist and Judy 1971). A study using pyridinol carbamate obtained a similar result (Sassard et al. 1979).

### 1.2.2.3. Metabolism of Carbamates

The purpose of metabolism in animals is to make xenobiotics more polar (Parke and Williams 1969; Timchalk 2006; Williams 1959). The metabolites of the xenobiotics could then be excreted from the body after metabolism. Since they are hydrophobic, carbamate compounds require efficient metabolism in the body to detoxicate them by making their metabolites hydrophilic (Ioannides 2002; Jakoby and Ziegler 1990; Parvez et al. 2001). Like other xenobiotics, the metabolism of carbamate compounds in animals involves both phase I and phase II reaction (Tang et al. 2006).

#### 1.2.2.3.1 Phase I Reactions
The major function of Phase I reactions is to form proper functional groups for Phase II derivitization (Tang et al. 2006). Generally speaking, Phase I reactions help to detoxicate xenobiotics and serve as a protective mechanism for animals (Hodgson and Goldstein 2001; Parkinson 2001). However, in some situations, this oxidation can increase the toxicity of xenobiotics, which facilitate the intoxication process. For example, both in vivo and in vitro, the metabolites of aldicarb, such as aldicarb sulfone and aldicarb sulfoxide, were more potent AChE inhibitors than aldicarb (Pelekis and Krishnan 1997).

For carbamate compounds, Phase I reactions are conducted by the mixed function oxidase system (MFO), which mainly catalyze oxidation (including epoxidation, dealkylation, sulfoxidation and hydroxylation) and hydrolysis (Tang et al. 2006). Liver is the major site responsible for these reactions in animals (Pekas and Paulson 1970), and the highest amount of MFO is in cellular microsomes (Gillette et al. 1969). During MFO reactions, NADPH serves as a cofactor and cytochrome P450 is the major electron transport enzyme system (Gillette et al. 1969; Brodie et al. 1958).

Oxidation is the most important metabolic process for carbamate compounds. Although there are many potential oxidation sites for carbamates, these sites could be categorized into two groups, which are either on ring or on side chain. Ring hydroxylation was first found in carbaryl (Dorough et al. 1963). In that paper, Dorough et al. incubated carbaryl with rat liver homogenate and found two ring hydroxylation products, which are 4-hydroxy- and 5-hydroxycarbaryl. Later, the same metabolism processes were also found for other carbamate insecticides, such as carbofuran (Dorough 1968; Metcalf et al. 1968). An example of ring hydroxylation is shown below (Figure 1-2):
Figure 1-2. Ring oxidation on 2-isopropoxyphenyl methylcarbamate (Propoxur). After MFOs' catalysis, propoxur is converted to 5-hydroxy-2-isopropoxyphenyl methylcarbamate.

Sometimes, this oxidation process may require an intermediate step called epoxidation (Hodgson and Goldstein 2001). For example, carbaryl ring hydroxylation requires an epoxide intermediate derivative (Figure 1-3).

Figure 1-3. Naphthalen-1-yl methylcarbamate is catalyzed by MFOs and metabolized into the epoxidation intermediate product, the naphtho[2,3-b]oxiren-3-yl methylcarbamate. MFOs further catalyze the transfer of the naphtho[2,3-b]oxiren-3-yl methylcarbamate to 6-hydroxynaphthalen-1-yl methylcarbamate.

Side chain oxidation can occur in many places, which may include N- and O-dealkylation, resulting from hydroxylation on the alkyl side chain, and hydrolysis. For example, N-dealkylation could proceed at two different sites in mexacarbate (Zectran or N-Methyl 4-dimethyamino-3,5-xylyl carbamate) (Figure 1-4).
Figure 1-4. MFOs-catalyzed mexacarbate N-dealkylation of side chain. MFOs catalyzed mexacarbate (4-(dimethylamino)-3,5-dimethylphenyl methylcarbamate) N-dealkylation on side chain, the metabolites were: 3,5-dimethyl-4-(methylamino)phenyl methylcarbamate (left), and 4-(dimethylamino)-3,5-dimethylphenyl carbamate (right).

An example of O-dealkylation can be found in propoxur (Figure 1-5). Other side chain oxidation includes hydroxylation on alkyl side chain and oxidative hydrolysis. Examples of these reactions are shown below in Figure 1-6.

Figure 1-5. MFOs-catalyzed propoxur O-dealkylation of the side chain, forming the metabolite: 2-hydroxyphenyl methylcarbamate.
As mentioned above, sulfoxidation is a unique oxidation process that can sometimes activate rather than detoxicate aldicarb in Phase I reactions. This oxidation happens only in a very specific group of carbamate compounds called thiocarbamates (Matsumura 1985; Tang et al. 2006). In the thiocarbamate family, all of its members have a thioether substituent in their chemical structures. Although S-dealkylation was also reported during Phase I reactions (Hodgson and Goldstein 2001), this reaction is believed to be rare in thiocarbamates (Tang et al. 2006). Aldicarb has been studied extensively (Kuhr and Dorough 1976; Pelekis and Krishnan 1997). Aldicarb oxidatized derivatives, such as aldicarb sulfoxide and aldicarb sulfone, become more potent AChE inhibitors (Oonnithan and Casida 1968). Foran et al. (1985) reported that the aldicarb sulfoxidation increased the toxicity of aldicarb by using in vivo Daphnia magna bioassay. When tested with on adult D. magna, the 48 hour IC₅₀ of aldicarb, aldicarb sulfoxide and aldicarb sulfone were 51 (45-59) ug/L, 43 (39-46) ug/L and 369 (320-430) ug/L, respectively. Similarly, the 48h LC₅₀ reading of aldicarb and aldicarb sulfoxide and aldicarb sulfone were 209 (173-265) ug/L, 103 (36-142) ug/L and 1124 (993-1320) ug/L, respectively. Some other sulfoxidation-activation examples are benfuracarb, carbosulfan,
mecarbam, and thiodicarb (Ecobichon 2001). However, this sulfoxidation activation so far has not been shown to apply to all thiocarbamates. This reaction is shown below (Figure 1-7).

![Chemical structure of sulfoxidation of aldicarb](image)

Figure 1-7. MFOs oxidize aldicarb [(E)-2-methyl-2-(methylthio)propanal O-methylcarbamoyl oxime] to aldicarb sulfoxone [(E)-2-methyl-2-(methylsulfonyl)propanal O-methylcarbamoyl oxime].

### 1.2.2.3.2. Phase II Reactions

After Phase I reactions, Phase II reactions will make further modification of carbamate compounds (Chen and Dorough 1979; Dorrough 1968; Hodgson and Rose 2004; Kuhr and Dorough 1976; Tang et al. 2006). Generally speaking, Phase II reactions are biosynthetic, and will add hydrophilic substituents to xenobiotics, which facilitates excretion (Dorough 1979; Kuhr and Dorough 1976; Tang et al. 2006). There are four kinds of Phase II enzymes, which are glutathione transferases (GST), glucuronosyl transferases, sulfotransferases (SULT) and acetyltransferases (Parkinson 2001; Sies and Packer 2005; WHO 1986). Glutathione (GSH) is the cofactor that GST uses to conjugate with xenobiotics (Kuhr and Dorough 1976). In vertebrates, glucosidation is conducted by uridine diphosphate glucuronosyltransferase, one member of the glucuronosyl transferases family, which uses glucuronic acid from uridine diphosphate glucuronic acid (UDPGA) (Dorough 1979; Tang et al. 2006). SULT and acetyltransferases catalyze sulfation and acetylation, respectively (Parkinson 2001). Glutathione sulfate, mercapturic acid or glucuronide are connected to the metabolites of carbamate compounds from Phase I reaction. All these conjugated xenobiotics
metabolites become more hydrophilic than their parent compounds, which facilitate their final excretion either through the kidney or feces (Dorough 1979; Parkinson 2001; Tang et al. 2006).

1.2.3. Pharmacodynamics & Pharmacokinetics of Carbamates Insecticides

1.2.3.1. The Acetylcholine System

1.2.3.1.1. Introduction of The Acetylcholine System

Acetylcholine (ACh) is a neurotransmitter present at both neuromuscular junction and nerve cholinergic synapses in mammals, but only in the central nervous system in insects (Callec 1985; Nation 2002). The serine hydrolase family includes acetylcholinesterase (AChE), carboxylesterases, butyrylcholinesterase (BChE), neuropathy target esterase (NTE) and other esterases (Marrs and Ballantyne 2004; Massoulié et al. 1993). The main physiological function of AChE is to quickly terminate the transmitter function of ACh and prevent toxic effects from over-accumulation of ACh at synaptic cleft (Fukuto 1990). After reviewing the gene sequence, secondary structure, and 3-D structure of AChE (Sussman et al. 1991), it is clear that although the amino acid sequence is not conserved among species (Wiesner et al. 2007), the overall structure of AChE is highly conserved and consists of three functional regions, which are the catalytic anionic site (CAS), peripheral anionic site (PAS) and a connecting gorge (Bourne et al. 1995; 1999). X-ray crystallography shows that the gorge is a long and deep structure that is lined by 14 conserved aromatic residues (Bourne et al. 1995; Sussman et al. 1991). The CAS is located at the bottom of the gorge, which has the catalytic triad, Ser203, His447 and Glu334 (Bourne et al. 1995; Sussman et al. 1991). There are also four other subsites near the CAS, which are the esteratic site (Pavlic 1975; Wilson 1951), oxyanion hole (Ordentlich et al. 1998), anionic subsite (Bucht et al. 1994) and the acyl pocket (Taylor and Radic 1994). The PAS (Asp74, Tyr124, Ser125, Trp286, Tyr337 and Tyr341) is located near the
opening of the gorge, which is believed to attract and facilitate ACh movement into the gorge (Massoulié et al. 1993; Sussman et al. 1991). Upon binding, the carbonyl group of ACh reacts with the CAS of AChE (Figure 1-8). This reaction rapidly generates two products: choline and acetylated AChE (Quinn 1987). As an intermediate product, acetylated AChE is unstable in water and is readily hydrolyzed (Quinn 1987; Taylor and Radic 1994). The final products of one reaction cycle (Figure 1-8) are choline and acetic acid (Quinn 1987; Taylor and Radic 1994).

![Figure 1-8. Acetylcholine hydrolysis by acetylcholinesterase.](image)

**1.2.3.1.2. Mode of Action And Pharmacokinetics of Carbamate and Organophosphate Compounds on the Acetylcholine System**

AChE reacts with carbamate compounds in a similar way as its catalytic hydrolysis of ACh (Figure 1-9) (Wilson et al. 1960). After diffusing into the gorge and reaching the catalytic site of AChE, carbamate compounds first form a non-colvent intermediate with AChE, which is a Michaelis Complex (Radic and Taylor 2006). The rate of this step is determined by two constants, which are the association rate constant, $k_1$ and the dissociation rate constant, $k_{-1}$. The equilibrium constant of intermediate product formation is $K_d$ (unit is M$^{-1}$, mM$^{-1}$, etc.) which equals $k_1/k_{-1}$ (Aldridge and Reiner 1972; Richardson 1992). The carbonyl group of the carbamate then attacks the serine hydroxyl group at the AChE catalytic site, forming the carbamoylated AChE and one leaving group. This serine carbamoylation step is reversible and $k_c$, the rate constant (units are min$^{-1}$, sec$^{-1}$, etc.), is used to describe the rate of this reaction step (Aldridge and Reiner 1972). Since most of the carbamate compounds (except the thiocarbamate group) can inhibit AChE without metabolic pre-
activation, once the carbamate compounds reach the catalytic site of AChE, the carbamoylation proceeds. Therefore, the rate of intermediate product formation determines the rate of the carbamoylation process and is directly correlated with the inhibitory capacity of carbamate, which means the $K_d$ value can be used to mostly describe the inhibitory activity.

![Diagram](image)

Figure 1-9. Diagram demonstrating the reaction between serine hydrolase (acetylcholinesterase) and a carbamate.

The bimolecular rate constant of inhibition, $k_i$ (mM$^{-1}$ min$^{-1}$), is used to quantify the inhibition potency of carbamate compounds (Aldridge and Reiner 1972; Main 1964). Because $k_i$ is equal to $k_c/K_d$, it describes both the site affinity and carbamoylation rate of AChE, and is specific for each carbamate compound (Fukuto 1990; Main 1964). The more potently a carbamate compound binds and the faster the carbamoylation rate is, the bigger the $k_i$ value will be (Fukuto 1990). Mathematically, the $k_i$ value is determined by using the equation that $ln(A_n/A_0) = -k_i *[Inh]*t$, where $A_n$ and $A_0$ are the remaining and initial activity of AChE, respectively, while $t$ is the time AChE interacts with carbamates (Radic and Taylor 2006). [Inh] means the concentration of AChE inhibitor. By replacing different chemical groups on carbamate compounds, the $k_i$ value is subject to change because both Michaelis Complex formation and carbamoylation rate are changed (Fukuto 1990; Sultatos 2006). This structure-activity property has been advantageous in dealing with different problems such as pesticide development (Sultatos 2006), which will be discussed later.

Finally, the carbamoylated AChE is reactivated through slow hydrolysis by water, which is characterized by the reactivation constant, $k_r$ (Aldridge and Reiner 1972; Fukuto 1990; Segel 1975).
Unlike acetylated AChE, the carbamoylated AChE is more stable and the decarbamoylation process usually takes about an hour (Fukuto 1990; Main 1980). Thus, acetylated AChE has a larger $k_r$ value than that of carbamoylated AChE (Fukuto 1990). Similar to AChE’s deacetylation process, the final products of one reaction cycle will not change in either quantity or quality of AChE (Thompson and Richardson 2004). Structure–activity property of carbamate can also regulate the rate of water hydrolysis step (Sultatos 2006). For example, compared to its corresponding $N,N$-dimethyl carbamate, $p$-nitrophenyl $N$-monomethyl carbamate has a 7 times larger $k_r$ value of $5.4 \times 10^6 \text{ M}^{-1}\text{min}^{-1}$ (Metcalf 1971). A detailed mechanism will be discussed later in this thesis.

Organophosphate compounds are another family of cholinesterase inhibitors. Although different in chemical structure, organophosphates share a similar AChE reaction process with carbamate compounds, and include navigating the gorge and AChE phosphorylation. However, the value for $k_r$ of organophosphate compound is extremely small (Richardson 1992), because phosphorylated AChE is extraordinarily stable and resistant to water hydrolysis. Compared to 1 hour water hydrolysis for carbamoylated AChE, water hydrolysis of phosphorylated AChE takes hours to days depending on the structure of the organophosphate compound (Main 1980). By using oxime antidotes, such as 2-PAM and TMB-4 (Wilson and Ginsburg 1955; Wilson et al. 1958), the phosphoryl-serine bond can be broken and dephosphorylation happens. This phenomenon shows mathematically as an increased $k_r$ value.

However, if the antidotes are not applied in time, aging may occur, which is a unique characteristic of phosphorylated AChE, but not carbamoylated AChE (Casida and Quistad 2004). The definition of aging is the dealkylation of phosphoryl substituent on the phosphorylated AChE, which strengthens the covalent bond and makes it impossible for water hydrolysis. It has been shown that this aging process is affected by the protonated His447 of the CAS, a glutamic acid
residue adjacent to the CAS, and a Trp-86 residue nearby the CAS (Shafferman et al. 1996; Viragh et al. 1997). Once aging occurs, even oxime antidotes can no longer break the phosphoryl-serine bond (Fukuto 1990). In this case, phosphorylated AChE is going to be permanently inhibited and only biological degradation can proceed.

1.2.3.1.3. Structure–Activity Relationships of Carbamate Insecticides on Acetylcholinesterase

As mentioned above, by changing the chemical substituents in appropriate places, the pharmacokinetic properties of carbamate compounds can be changed. Steric property is very important for carbamate compounds to access the catalytic site before a covalent bond is formed. Normally, it is believed that the best structure for carbamate should be close to that of ACh (Sultatos 2006; Kolbezen et al. 1954). One example is m-isomer of tert-butylphenylmethylcarbamate, which has been shown to have a highly effective anticholinesterase capacity (Kolbezen et al. 1954). As mentioned before, modification of the carbamate chemical structure at appropriate sites can change both carbamoylation and decarbamoylation rate, which mathematically shows an altered $k_c$, $K_d$, $k_r$ and $k_i$ value. For insecticidal usage, the optimum carbamate compound should have the highest reactivity and selectivity on target insect species. In addition, it is also important for carbamate compounds to possess appropriate physicochemical properties to penetrate cuticle of blood brain barrier.

$N$-alkyl substituents are responsible for the reaction rate of carbamoylation or in other words, determine the $k_c$, $K_d$, $k_r$ value. $N,N$-dimethyl carbamate is less reactive than $N$-monomethyl carbamate because the bulky substituent of the alkyl group on nitrogen blocks the access to catalytic site in the AChE’s gorge (Thompson and Richardson 2004). This will cause a decreased rate of binding and reaction and lowered $k_c$, $K_d$, $k_r$ value. In this case, as a weak AChE inhibitor with
slower reaction rate and less significant biological effect, \(N,N\)-dimethyl carbamate compounds are more often used for clinical therapy purposes, while \(N\)-monomethyl carbamate compounds are good insecticides. Representative examples are neostigmine (Komloova et al. 2010) and bendiocarb (PAN Pesticides Database 2000b), which are dimethyl- and monomethyl carbamate compounds respectively. As a dimethyl carbamate compound, neostigmine has a smaller \(k_c\), bigger \(K_d\), and smaller \(k_r\) compared to that of the monomethyl carbamate compounds. On the one hand, the small \(k_c\) and bigger \(K_d\) would less likely to cause an acute toxicity on patients who take neostigmine. On the other hand, the smaller \(k_r\) value allows a persistent AChE inhibition at a moderate dose, which is easier for patients taking neostigmine. However, as an insecticide, bendiocarb displays a bigger \(k_c\) and smaller \(K_d\), which cause potent effects on the insect.

\(N\)-alkyl substituents also account greatly for the rate of the decarbamoylation step. Research has showed that different carbamoylated forms of AChE has different susceptibility to hydrolysis (O’Brien 1967). This susceptibility order of carbamoylated AChE is known to be \(\text{Enz–COCH}_3 \gg \text{Enz–C(O)NH}_2 > \text{Enz–C(O)NHCH}_3 > \text{Enz–C(O)N(CH}_3)_2\) (O’Brien 1967). Thus, \(N\)-alkyl substituents play an important role in carbamate structure-activity relationship, which greatly determines the pharmacodynamics of each carbamate compound.

Generally, in a phenyl carbamate compound, for a given alkyl group substituent (but not the alkoxy groups), the 3- or \textit{meta} position produces the most active AChE inhibitor, which is followed by the ortho position and \textit{para} position. Research has showed that the meta-position provides the optimum distance between the carbonyl group and substituent group (Kuhr and Dorough 1976). Either increasing or decreasing this distance lowers the inhibitory potency. The inhibitory potency order of alky group substituent are known to be \textit{meta} \(> \text{ortho} > \text{para}\) (Kuhr and Dorough 1976). In addition, at a given position on the phenyl ring, increasing the substituent chain size, from 1 carbon
to 3 carbons, can increase the anticholinesterase capacity. However, any branched chain, which was larger than 4 carbons, is generally going to reduce the inhibitory capacity (Kuhr and Dorough 1976). In conclusion, chemical modification of carbamate compounds will regulate its activity, and appropriate design of carbamate compound will maximize the desired result and minimize undesirable effects.

1.2.3.2. Neurotoxic Esterase and OPIDN

1.2.3.2.1. Introduction of Neurotoxic Esterase and OPIDN

Neurotoxic esterase (NTE) is a unique serine hydrolase that responds to different serine covalent inhibitors. NTE is sensitive to neuropathic organophosphate compounds, such as \(N,N'-\)diisopropyl-phosphorodiamidofluoridate (mipafox) and diisopropylfluorophosphate (DFP) (Figure 1-10) (Johnson 1982).

![Figure 1-10. Structure of Mipafox (N,N'-diisopropylphosphorodiamidic fluoride) (left) and Diisopropylfluorophosphate (DFP) (diisopropyl phosphorofluoridate) (right).](image)

Although distributed in a variety of tissues, such as placenta, spleen, etc. (Williams 1983), NTE has the highest reactivity and quantity in brain in all species that have been examined (Richardson 1992). At the subcellular level, NTE is mainly defined as a membrane associated protein that catalyzes the phosphatidylcholine to glycerophosphocholine deacylation (Richardson 1992). By comparing NTE gene sequences through species, it is known that vertebrate animals,
invertebrate animals, and microorganisms share highly conserved DNA coding sequence and reading frame organization (Lush et al. 1998). In insects such as *Drosophila melanogaster*, an NTE homologue called Swiss Cheese Protein (SWS) exists (Kretzschmar et al. 1997). After more than 70% NTE inhibition, there will be a distal swelling and dying back degeneration of motor and sensory peripheral nerves in both peripheral and central (spinal cord) nerves system, which is termed organophosphate-induced delayed neuropathy (OPIDN) (Lotti and Johnson 1980). In insects, the inhibition of SWS causes the same histological changes, presenting as glial over-wrapping and neurodegeneration (Kretzschmar et al. 1997).

The first human cases of OPIDN occurred in 1920 in the United States because of OP poisoning (Smith et al. 1930). It was reported that after consumption of a beverage called “Jamaica Ginger,” more than 30,000 people developed a specific “Ginger paralysis” which made them unable to use their hands and feet. Later, a chemical named tri-ơ-tolyl phosphate (TOCP) was isolated from this drink, which was shown to be responsible for Ginger paralysis (Goldfain 1930; Smith et al. 1930). Later results strongly suggested that NTE is the target site for TOCP, the inhibition of which was the major underlying mechanism of OPIDN (Johnson 1974; 1990). Subsequently, more neuropathic organophosphate compounds were found and NTE inhibition that correlated with OPIDN was demonstrated in other animal species, such as rodent, feline, and avian. After organophosphate dosing, they all presented the classical OPIDN patho-histological and behavior changes (Bouldin and Cavanagh 1979b; Carboni et al. 1992; Drakontides et al. 1982; Stumpf et al. 1989; Veronesi 1984). Particularly, compared to the mammalian rodent model, the avian (chicken) model expressed the highest sensitivity to neuropathic organophosphate compounds and is the best association model between nerve lesions and locomotion deficits (Dudek et al. 1979; Dudek and Richardson 1982).
1.2.3.2. Mode of Action and Pharmacokinetics of Carbamate and Organophosphate Compounds on NTE

As a serine hydrolase, all carbamate and organophosphate compounds could potentially access and covalently bind to the catalytic site of NTE, resulting in its functional inhibition (Clothier and Johnson 1980; Johnson 1982). However, among all the NTE inhibitors, scientists used to believe only certain organophosphate compounds can induce OPIDN, which are phosphates, phosphonates and phosphoramidates (Glynn 2006; Moretto and Lotti 2006) (Table 1-1.). Phosphinates, on the other hand, were believed to be safe for NTE (Johnson 1974, Glynn and Johnson 2001) (Table 1-1.). More recently, some carbamate compounds have also been shown to cause delayed neuropathy (Lotti and Moretto 2006; Randall et al. 1997).
<table>
<thead>
<tr>
<th>Neuropathic Organophosphate Compounds</th>
<th>Non-neuropathic Organophosphate Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name</strong></td>
<td><strong>Structure</strong></td>
</tr>
<tr>
<td>Phosphates</td>
<td>$\begin{array}{l} \text{R}_1 \text{O} \text{P} \text{O} \text{X} \ \text{R}_2 \text{O} \text{P} \text{O} \text{X} \end{array}$</td>
</tr>
<tr>
<td></td>
<td><img src="image1" alt="Diethyl 2,2-dichlorovinyl phosphate" /></td>
</tr>
<tr>
<td>Phosphonates</td>
<td>$\begin{array}{l} \text{R}_1 \text{O} \text{P} \text{X} \ \text{R}_2 \text{O} \text{P} \text{X} \end{array}$</td>
</tr>
<tr>
<td></td>
<td><img src="image3" alt="Leptophos oxam" /></td>
</tr>
<tr>
<td>Phosphorimidates</td>
<td>$\begin{array}{l} \text{R}_1 \text{O} \text{P} \text{N} \text{X} \ \text{R}_2 \text{O} \text{P} \text{N} \text{X} \end{array}$</td>
</tr>
<tr>
<td></td>
<td><img src="image4" alt="Methamidiphos" /></td>
</tr>
<tr>
<td>Others</td>
<td>$\begin{array}{l} \text{N} \text{C} \text{O} \text{P} \text{S} \ \text{N} \text{C} \text{O} \text{P} \text{S} \end{array}$</td>
</tr>
<tr>
<td></td>
<td><img src="image5" alt="Cyanophosphor" /></td>
</tr>
<tr>
<td></td>
<td><img src="image6" alt="Cloxynylelis" /></td>
</tr>
</tbody>
</table>
The OPIDN-inducing organophosphate compounds are called neuropathic OPs. Organophosphate compounds such as paraoxon are called non-neuropathic OP because they do not inhibit NTE (Glynn 2006). These non-neuropathic OPs, as well as non-OPIDN inducing carbamate compounds (Johnson 1970), can even protect against OPIDN incidence if administrated to experimental animals before neuropathic organophosphate compounds (Moretto and Lotti 2006). It is believed that neuropathic organophosphate compounds undergo aging after covalent binding, while non-neuropathic covalent NTE inhibitors cannot (Davis et al. 1985; Clothier and Johnson 1980; Johnson 1982; Lotti and Johnson 1980). The aged NTE loses its physiological function, thus leading to axonal degeneration.

For carbamate compounds, the pharmacodynamics of NTE inhibition is the similar to that of AChE inhibition (Moretto and Lotti 2006). After arriving at the catalytic site of NTE, they bind to NTE and form a Michaelis Complex, producing the carbamoylated NTE. Similar to carbamoylated AChE, carbamoylated NTE is more stable than the NTE complex with its natural substrate. However, unlike phosphorylated NTE, carbamoylated NTE can begin to gradually hydrolyze, which usually takes hours, but will eventually restore the active NTE (Moretto and Lotti 2006). All kinetic constants described are the same as that of the AChE carbamoylation processes, and have the same meaning.

For organophosphates, the reaction procedures are the same as that AChE phosphorylation in the early steps (Figure 1-11). First, organophosphate compounds attack the NTE active site, forming a Michaelis Complex, resulting in production of phosphorylated NTE. All kinetic constants described are the similar to those of the AChE carbamoylation processes, and have the same meaning.
Figure 1-11. Diagram demonstrating the reaction between serine hydrolase (NTE) and different organophosphate compounds. When reacting with neuropathic OP, neurotoxic esterase is phosphorylated and follows the aging pathway. When reacting with non-neuropathic OP, neurotoxic esterase is phosphorylated first, but reactivated later.

However, unlike that of NTE carbamoylation, the pharmacokinetic step of phosphorylated NTE splits into two separate pathways because of the aging capacity difference between neuropathic OP and non-neuropathic OP (Glynn 2006; Moretto and Lotti 2006). For neuropathic OP, which usually contain at least one –O- or –NH- substituents linking to phosphorus, further dealkylation on one site on the side chain forms an aged NTE (Glynn and Johnson 2001). Aged NTE does not allow the de-phosphorylation process and both its reactivity and reaction is blocked (Glynn and Johnson 2001). The non-neuropathic OP does not proceed to the aging pathway and allows the slow hydrolysis of phosphoryl bond, which eventually releases the activated NTE. This reactivation still takes days before NTE gets reactivated because phosphorylated NTE is stable and is resistant to water hydrolysis. In this case, \( k_r \) value equals to 0 for neuropathic OP while \( k_r' \) value is small compared to that of non-neuropathic carbamate compounds.

1.2.4. Toxicty

1.2.4.1. Toxicity of Carbamate Compounds in Human
Potent carbamate compounds can cause intoxication in many organs based on the exposure dose and time. After a chronic exposure to carbamate compounds, multiple organ functions and histological organization may be affected, including the central and peripheral nerve, cardiovascular, pulmonary, dermal, endocrine and reproductive systems (Gupta 2006). In situations of acute carbamate intoxication, symptoms are consistent with cholinergic system over-stimulation and expressed as a combination of both nicotinic and muscarinic effects. The classical symptoms are described in humans as SLUDGE (salivation, lacrimation, urination, defecation, gastrointestinal distress and emesis) (Moser 1995; 1999; Moser et al. 1988). In addition, as a serine hydrolase, NTE could also be inhibited by certain carbamate compounds, which eventually cause OPIDN (Lotti and Moretto 2006; Randall et al. 1997).

1.2.4.2. Methods to Test Reactivity of Carbamate Compounds

1.2.4.2.1. Ellman Assay for Cholinesterase Reactivity

The most common method for detecting cholinesterase reactivity was introduced by Ellman et al. (1961), which is a chemical cascade reaction based on the Ellman reagent. Ellman reagent, or 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), is a water soluble, colorless, crystalline compound at room temperature. In solution, it is very sensitive to exposure to free sulfhydryl groups. After reaction with the sulfhydryl group, two products are formed, which are a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB). TNB is yellow colored and can be measured by a spectrometer with absorbance at 405 nm and AChE inhibition is quantified by reduced levels of yellow color.

The free sulfhydryl groups in Ellman Assay come from the product of thiocholine ester substrates of AChE. Just like the steps of ACh hydrolysis, AChE hydrolyzes acetylthiocholine ester and produces free sulfhydryl groups in solution. There are three classical thiocholine ester substrates
that are still widely used. They are PTCh (propionylthiocholine iodide), BTCh (butyrylthiocholine iodide) and ATCh (acetylthiocholine iodide) (Yamada et al. 2001). Because PTCH, BTCH and ATCh have different properties and reactivity rates, they are used selectively in different cholinesterase testing methods. For human AChE in Ellman Assay, ATCh is the preferred substrate (Reiner et al. 2000). The detailed chemical reaction involved in Ellman Assay is presented below (Figure 1-12):

\[
\begin{align*}
\text{ATCh} & \quad + \quad \text{AChE} \quad \xrightarrow{\text{H}_2\text{O}} \quad \text{Acetic acid} \quad + \quad \text{Thiocholine} \\
2-(\text{acetylthio})-N,N,N-\text{trimethylethanaminium} & \quad + \quad 2 \text{H}^+
\end{align*}
\]

**Step 1. ATCh hydrolysis**

\[
\begin{align*}
\text{DTNB} & \quad \xrightarrow{+ \text{ATCh}} \quad \text{Disulfide} \\
2\text{-carboxy-4-((3-carboxy-4-nitrophenyl)disulfanyl)benzoate} & \quad + \quad 3\text{-carboxy-4-nitrobenzenethiolate}
\end{align*}
\]

**Step 2. Disulfide formation**

Figure 1-12. Ellman assay reaction procedures. Step 1. ATCh is added to a solution containing AChE. AChE will hydrolyze ATCh and generate two products, which are thiocholine and acetic acid. Step 2. DTNB reacts with thiocholine to form TNB, which shows a measurable yellow color.

1.2.4.2.2. Neurotoxic Esterase Assay

NTE assay was developed and improved by Johnson (Johnson 1969; 1975; 1977). Usually, paraoxon and mipafox will be used as a general esterase inhibitor and neuropathic organophosphate
compound, respectively. After pre-incubation with paraoxon and paraoxon + mipafox respectively, NTE activity could be obtained by detecting the serine hydrolase reactivity difference between these two samples. The substrate phenyl valerate is hydrolyzed to liberate phenol by NTE. Next, by adding an alkaline solution of potassium ferricyanide, this liberated phenol will react with 4-aminoantipyrine, forming the final product called N-(1, 4-benzoquinoneimine)-antipyrine, which is yellow colored and has maximum absorbance at 510 nm wavelength. Finally, the experiment is stopped by putting the detergent sodium dodecylsulfate (SDS) into the solution (Johnson 1977).

Chapter 2 Experimental Design and Methods

2.1. Materials

Three commercial carbamate insecticides, bendiocarb, carbofuran, and propoxur were donated by or purchased from Nor-AM Chemical Company, Chem-Service, and Mobay Corporation, respectively. All chemicals were at analytical grade, which was >95% purity. All novel carbamate insecticides were synthesized and characterized in the laboratory of Dr. Paul R. Carlier, Department of Chemistry, Virginia Polytechnic Institute and State University. The structures of these novel carbamate insecticides are shown below (Table 2-1). Acetylthiocholine iodide (ATCh), 5,5-dithio-2,2-nitrobenzoic acid (DTNB), bovine serum albumin (BSA), and TritonX-100 were purchased from Sigma (Sigma-Aldrich Corporation, St. Louis, MO, USA). All chemicals were at analytical grade. Paraoxon and mipafox were obtained from the laboratory of Dr. Marion F. Ehrich.
Table 2-1. Structure name and chemical name of novel carbamate insecticides that were tested in this study.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Name</th>
<th>Chemical Name</th>
<th>Structure</th>
<th>Name</th>
<th>Chemical Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure" /></td>
<td>PRC 331</td>
<td>3-(tert-butyl)phenyl methylcarbamate</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>PRC 391</td>
<td>2-(isobutylthio)phenyl methylcarbamate</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure" /></td>
<td>PRC 337</td>
<td>2-((2-methylallyl)thio)phenyl methylcarbamate</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>PRC 407</td>
<td>2-((2-methylbutyl)thio)phenyl methylcarbamate</td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure" /></td>
<td>PRC 387</td>
<td>3-(trimethylsilyl)phenyl methylcarbamate</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>PRC 408</td>
<td>2-((2-ethylbutyl)thio)phenyl methylcarbamate</td>
</tr>
<tr>
<td><img src="image7.png" alt="Structure" /></td>
<td>PRC 388</td>
<td>3-(ethylidimethylsilyl)phenyl methylcarbamate</td>
<td><img src="image8.png" alt="Structure" /></td>
<td>PRC 421</td>
<td>2-(2-ethylbutoxy)phenyl methylcarbamate</td>
</tr>
</tbody>
</table>

This table demonstrated the structure, general name and chemical name of novel carbanates that were tested in this study.
2.2. Animal and Sample Preparation

2.2.1. Recombinant Human and Mouse AChE

Recombinant human and mouse AChE were kindly donated by Dr. Fredrik Ekström, of the Swedish Defense Research Agency. The detailed cloning and expression method can be obtained elsewhere (Ekstrom et al. 2006). After receiving the enzyme in original concentration, a 50-fold dilution and 30-fold dilution was made for recombinant mouse and human AChE, respectively, and aliquots were held at -80 °C until use. Each 20 ul of mouse AChE was mixed with 980 ul ice cold sodium hydrogen phosphate, pH 7.8, containing 3% Triton-x-100 (Sigma, USA). Each 25 ul human AChE was mixed with 725 ul ice cold sodium hydrogen phosphate, pH 7.8, containing 3% Triton-x-100 (Sigma) was added.

2.2.2. Hen Brain Homogenate

Hen brain samples were obtained from Dr. Marion Ehrich. Hens were sacrificed by cervical dislocation, the brain was dissected rapidly on ice, and kept in ice-cold artificial cerebrospinal fluid (ACSF, in mM: NaCl, 124.0; NaHOC₃, 25.0; D-glucose, 10.0; KCl, 3.4; KH₂PO₄, 1.2; MgSO₄, 1.0; CaCl₂, 2.5; pH 7.4). Each 10 mg hen brain was homogenated in 25 ml ice cold sodium hydrogen phosphate, pH 7.8, containing 3% Triton-x-100. This sample was then stored in a -80 °C freezer for use in NTE assays. For Ellman assay, this brain homogenate sample was further centrifuged at 10,000 RPM, 5000 x g, 4 °C for 15 minutes using a Sorvall Fresco refrigerated centrifuge and all the supernatants were transferred to a clean 1.5 Eppendorf tube and stored in a -80 °C freezer.

2.2.3. *Electrophorus electricus* Acetylcholinesterase

Recombinant *E. electricus* AChE (400 U/mg protein) was purchased from Sigma–Aldrich (USA). The enzyme was diluted in 1% ice cold BSA solution in a ratio of 1.53 mg AChE : 1.68 ml
BSA, resulting in a final concentration of 600 U/ml. An additional 801 fold dilution was performed (1.2 ul enzyme solution was added into 960 ul ice cold sodium hydrogen phosphate, pH 7.8, containing 3% Triton-x-100. All enzymes preparations were then kept in a -80°C freezer.

2.2.4. Torpedo californica Homogenate

Tissue from the electroplaque organ of *T. californica* was obtained from Aquatic Research Consultants (San Pedro, CA). After rinsing with ice cold sodium hydrogen phosphate, the electroplaque organ was cut into small pieces and 100 mg electroplaque organ tissue was homogenized in 5ml ice cold sodium hydrogen phosphate, pH 7.8, containing 3% Triton-x-100. The homogenates were then centrifuged at 10,000 RPM, 5000 x g, 4 °C for 15 minutes using a Sorvall Fresco refrigerated centrifuge. The supernatants were removed and transferred to a clean 1.5 Eppendorf tube and stored in a -80 °C freezer.

2.2.5. Daphnia magna Homogenate

Living *Daphnia magna* were purchased from Carolina Biological Supply Company (Burlington, NC). Ninety *D. magna* were cultured in 1 gallon spring water and fed every other day, with food supplied by the *D. magna* culture kits from Carolina Biological Supply Company. Natural illumination was used and the light condition was about 12-12 h light-dark period. Culture kits were kept at room temperature, from 20 °C to 25 °C. Thirty *D. magna* were homogenized in 10 ml tubes with ice cold sodium hydrogen phosphate, pH 7.8, containing 3% Triton-x-100. Then the homogenates were centrifuged at 10,000 RPM, 5000 x g, 4 °C for 15 minutes using a Sorvall Fresco refrigerated centrifuge. The supernatants were removed and transferred to a clean 1.5 Eppendorf tube and stored in a -80 °C freezer.
2.2.6. *Anopheles gambiae* Homogenate

Frozen female *An. gambiae* was obtained from *An. gambiae* lab culture. Ten *An. gambiae* were homogenized in 10 ml tubes with ice cold sodium hydrogen phosphate, pH 7.8, containing 3% Triton-x-100. The homogenates were centrifuged at 10,000 RPM, 5000 x g, 4 °C for 15 minutes using a Sorvall Fresco refrigerated centrifuge. The supernatants were taken out and transferred to a clean 1.5 Eppendorf tube and stored in a -80 °C freezer.

2.3. Ellman Assay

Ellman assay (Ellman et al. 1961) was used to measure AChE activity. The 96 well microtiter plate reading spectrophotometer used for Ellman assay is from DYNEX Technologies, Chantilly, Virginia. Enzyme was mixed with 0.1 M sodium hydrogen phosphate buffer, pH 7.8, with six different inhibitors concentration. Each data set was replicated in triplicate. After 10 min preincubation of AChE with inhibitor, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and acetylthiocholine (ATCh) were added into the well. The hydrolysis of Ellman assay agents was determined in the same way in the absence of enzyme, which was taken as blank and deducted from all wells. The plate was read at 405 nm wavelength using a spectrophotometer for 10 min and a raw data sheet was obtained, which indicated the optical density of each well on the microtiter plate.

Then the raw data sheet of the Ellman Assay was processed in Excel™ software (Microsoft, Redmond, Washington). The activity remaining equation was used and shown below:

\[
\text{Remaining activity (\%)} = \frac{(A_n - A_{blank})}{A_0} \times 100\% 
\]

Where \(A_n\) = absorption of each well with carbamate at different concentration

\(A_{blank}\) = mean value of absorption of blank wells

\(A_0\) = average value of absorption of control wells (i.e., solvent without carbamate)
All data were then exported to GraphPad Prism™ 5.0 (GraphPad Software, San Diego, CA, USA) for further statistical analysis.

2.3.1. Dilution Methods for Ellman Assay

The AChE inhibitors were diluted into 6 different concentrations. Two dilution methods for inhibitors were used, which were: Method 1; a series dilution with a constant DMSO (DWCD) concentrations and Method 2; a series dilution with various DMSO (DW/OCD) concentration. The detailed steps are described below.

2.3.1.1 Method 1. A Series Dilution with a Constant DMSO (DWCD) Concentrations Procedures for AChE Assays

A 0.1 M AChE inhibitor stock solution was made freshly in DMSO before each experiment. A 10 ul aliquot was placed in 990 ul sodium hydrogen phosphate buffer, resulting in a 1 mM inhibitor solution. Then, 100 ul was removed and transferred to 900 ul hydrogen phosphate buffer, which diluted the inhibitor concentration by 10 fold. This series of 10 fold dilutions (5 times in all) provides an inhibitor solution range from 0.1mM to 10 nM. On the plate, another 10 fold dilution was performed, which made the final inhibitor concentration from 10 uM to 1 nM. The DMSO concentration varied in this case, which was 1%, 0.1%, 0.01%, 0.001%, 0.0001%, 0.00001% for aliquot 1 to 6. The detailed diagram is shown below (Figure 2-1):
Figure 2-1. The procedure of dilution without constant DMSO concentration method for Ellman assay. The aliquot (a) represented AChE inhibitor at 0.1 M concentration in DMSO. First, 10 ul was transferred from the stock solution in the aliquot (a) into the aliquot (b) containing 990 ul sodium hydrogen phosphate buffer, which diluted the stock solution by 100 fold. Then 100 ul from aliquot (b) was transferred into the aliquot (c) with 900 ul sodium hydrogen phosphate buffer, which further diluted the solution by 10 fold. Repeating this step 5 times yields a series of AChE inhibitor solutions with a 10 fold difference in adjacent aliquots. The final concentration in aliquot (b) and (c) ranged from 1 mM to 10 nM.

2.3.1.2. Method 2. A Series Dilution with Various DMSO (DW/OCD) Concentrations

Procedures for AChE Assays

A stock solution of 0.1 M inhibitor was made freshly in DMSO before each experiment. Then, 5 ul inhibitor solution was removed and placed in 45 ul 100% DMSO. This step was repeated 4 more times and the final products were 5 inhibitor stock solutions with a 10 fold concentration difference. Then a 10 ul inhibitor solution was transferred into 990 ul sodium hydrogen phosphate buffer. The final concentration of inhibitor ranged from 1 mM to 10 nM and 0.1 mM to 1 nM in the final aliquot and on the 96-well plate, respectively; the dilution series contained 10 fold differences. The DMSO concentration was a constant 1% in the aliquot (c) and 0.1% on plate. A detailed diagram is shown below (Figure 2-2):
2.4. Neuropathy Target Esterase (NTE) Assay

The NTE assay was performed by the procedures of Johnson (1977). Initially, different test chemicals, such as carbamates, diisopropylphosphorofluoridate (DFP), and DMSO alone, were incubated with hen brain homogenate for 20 min at room temperature. Then the samples were incubated with paraoxon (50 μM) only or paraoxon (50 μM) plus mipafox (6.4 μM) for 20 min in the assay buffer (50 mM Tris-0.2 mM EDTA, pH 8.0) at 37 °C. Following incubation with phenyl valerate (0.3 mM) for 15 min at 37 °C, the released phenol was analyzed by coupling with 0.4% potassium ferricyanide and SDS-aminoantipyrine (de-ionized water contains 5% sodium lauryl sulfate, 0.02% 4-aminoantipyrine, 0.50 M Tris) and measured in the spectrophotometer at 510 nm.
The value from the paraoxon only group (inhibit esterase other than NTE) was deducted from the paraoxon + mipafox group (which inhibited all esterases), giving the activity of NTE. Each remaining NTE activity value was estimated from at least 5 replicates.

In the NTE assay Excel™ spreadsheet, four sets of absorbance value were obtained from each 96 well plate, which were $A_{\text{blank}}$, $A_{\text{control}}$, $A_{\text{paraoxon}}$ and $A_{\text{mipafox+paraoxon}}$. The remaining activity efficacy was obtained by using the equation shown below:

$$\text{Remaining activity efficacy (\%) } = \frac{(A_{\text{paraoxon}} - A_{\text{mipafox+paraoxon}}) - A_{\text{blank}}}{A_{\text{control}}}$$

Where $A_{\text{blank}}$ = mean value of absorption of blank well

$A_{\text{control}}$ = mean value of absorption of well where sample was only incubated with solvent DMSO

$A_{\text{paraoxon}}$ = absorption of each well incubated with paraoxon and carbamate

$A_{\text{mipafox+paraoxon}}$ = absorption of each well incubated with paraoxon, mipafox and carbamate

All calculations were done in Excel and exported to GraphPad Prism™ 5.0 (GraphPad Software, San Diego, CA, USA) later for further statistical analysis.

2.5. In vivo D. magna Aquatic Toxicity Bioassay (LC$_{50}$)

Ten Adult D. magna were put in 10 ml of spring water (pH 7.3 to 7.8), which come with the D. magna culture kits (Carolina Biological Supply Company). Six concentrations of each carbamate as well as one control group using solvent only were used for each experiment. Serial dilution of carbamate insecticides from 0.1 M stock solution was used to make all dosing solutions; final concentrations ranged from 10mM to 0.1nM. After 24 h, the mortality rate was read in all containers.

Abbott’s formula (Abbott 1925) was used to correct bioassay data for control mortality. The equation was shown below.
Where \( P_{\text{exp}} \) = the survival number of \( D. \ magn\a in \) carbamate containing container

\( P_{\text{control}} \) = the survival number of \( D. \ magn\a in \) carbamate-free (with DMSO) container

All calculation was done in Excel™ and exported to GraphPad Prism™ 5.0 (GraphPad Software, San Diego, CA, USA) later for further statistical analysis.

2.6. Statistical Analysis

GraphPad Prism™ 5.0 (GraphPad Software, San Diego, CA, USA) software was used on all data for statistical test.

For the Ellman assay and the \( in \ vivo \) \( D. \ magn\a \) bioassay, the \( IC_{50} \) and \( LC_{50} \) values, respectively, were obtained from the sigmoid curve generated by using the nonlinear regression equation as shown below:

\[
y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1+10^{((\log(\text{LC}_{50}) - x) \times \text{Hill slope})})}
\]

Where \( x \) = the logarithm of the concentration of carbamate

\( y \) = the % of control response

Then, either the \( IC_{50} \) or \( LC_{50} \) mean value were obtained from replicate assays and compared by using a two-tailed student \( t \)-test. Significant difference was defined by whether \( P \) value was less than 0.05.

For enzyme pharmacological comparisons, all \( IC_{50} \) data were first converted into their corresponding \( \log_{10} \) value. After being imported into the GraphPad Prism™ 5.0, the data were fitted into a line with linear regression. \( R^2 \) (the coefficient of determination) was obtained. Then the data were further compared using one-way ANOVA with Dunnett’s test for significance between different groups (\( P < 0.05 \)).
For NTE results, single point enzyme activity of controls and pesticide-exposed groups was used to calculate a group mean for each carbamate insecticide and compared using an ANOVA with Dunnett’s test for significance between treatment means and a control ($P < 0.05$).

2.7. Multiple Sequence Alignment

Multiple sequence alignment of ‘acetylcholinesterase protein sequence’ was downloaded in FASTA format from the NCBI (The National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) for mouse, human, hen, $T. \text{californica}$ and $E. \text{electricus}$. The protein sequence used was the precursor of the AChE. All the protein sequences were then imported into the ClustalW2EMBL-EBI (The European Molecular Biology Laboratory - European Bioinformatics Institute, http://www.ebi.ac.uk/Tools/msa/clustalw2/). The results, including multiple sequence alignment, results summary, and phylogram tree were automatically generated.
Chapter 3: Results

3.1. Assay Validation for Comparison with Previous Results

Initially, I compared my results with historical data, to verify the accuracy of procedures and equipment. I used three different AChE enzyme sources, which were untreated P1 or P2 (pelleted after one or two centrifugations) male ICR mouse brain cortex homogenate, recombinant mouse, and recombinant human AChE. I tested them with nine commercial carbamate insecticides, using Method 1, DW/OCD method: series dilution without constant DMSO concentrations, which varied from 0.1% to 0.00001%.
Figure 3-1. Ellman Assay results using dilution without constant DMSO concentration. (a) One example of data from the Ellman Assay. Methomyl was tested on untreated male ICR mouse brain homogenate. Six data points were input into GraphPad Prism\textsuperscript{TM} 5.0 and a sigmoidal curve was obtained. IC\textsubscript{50}, Hill slope and R\textsuperscript{2} were automatically generated by this software. (b) IC\textsubscript{50} values were obtained from 9 commercial carbamate insecticides on three different non-target vertebrate AChE sources. The data of each carbamate were repeated at least 4 times. One-way ANOVA test (Turkey) was conducted and no significant difference was detected.
Table 3-1. IC\textsubscript{50} obtained from 9 commercial carbamate insecticides tested on untreated male ICR mouse brain cortex homogenate, recombinant mouse, and recombinant human acetylcholinesterase. Dilution without constant DMSO concentration dilution method was used for Ellman assay dilution.

<table>
<thead>
<tr>
<th>IC\textsubscript{50} (nM)</th>
<th>Aldicarb</th>
<th>Bendiocarb</th>
<th>Carbaryl</th>
<th>Carbofuran</th>
<th>Methomyl</th>
<th>Propoxur</th>
<th>Pirimicarb</th>
<th>Thiodicarb</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICR mouse brain cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>2874</td>
<td>208.6</td>
<td>3274</td>
<td>63.06</td>
<td>1602</td>
<td>1649</td>
<td>4665</td>
<td>2970</td>
</tr>
<tr>
<td>S.E.M</td>
<td>± 69</td>
<td>± 32</td>
<td>± 224.8</td>
<td>± 2.97</td>
<td>± 150.2</td>
<td>± 111.7</td>
<td>± 761.1</td>
<td>± 545.3</td>
</tr>
<tr>
<td>Hill slope</td>
<td>-1.12</td>
<td>-1.26</td>
<td>-1.15</td>
<td>-1.09</td>
<td>-1.13</td>
<td>-1.13</td>
<td>-1.18</td>
<td>-0.68</td>
</tr>
<tr>
<td>(R^2)</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Recombinant mouse AChE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1808</td>
<td>214</td>
<td>3922</td>
<td>57</td>
<td>808.1</td>
<td>1183</td>
<td>3405</td>
<td>3511</td>
</tr>
<tr>
<td>S.E.M</td>
<td>± 218.7</td>
<td>± 6.398</td>
<td>± 139.9</td>
<td>± 3.76</td>
<td>± 50.79</td>
<td>± 73.74</td>
<td>± 146.8</td>
<td>± 188.2</td>
</tr>
<tr>
<td>Hill slope</td>
<td>-1.07</td>
<td>-1.28</td>
<td>-1.03</td>
<td>-1.21</td>
<td>-1.13</td>
<td>-1.29</td>
<td>-1.22</td>
<td>-0.85</td>
</tr>
<tr>
<td>(R^2)</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Recombinant human AChE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>7317</td>
<td>359</td>
<td>3292</td>
<td>97.9</td>
<td>1583</td>
<td>2094</td>
<td>7358</td>
<td>4413</td>
</tr>
<tr>
<td>S.E.M</td>
<td>± 297.8</td>
<td>± 16.23</td>
<td>± 89.44</td>
<td>± 6.88</td>
<td>± 60.19</td>
<td>± 40.29</td>
<td>± 558</td>
<td>± 165.2</td>
</tr>
<tr>
<td>Hill slope</td>
<td>-1.04</td>
<td>-1.28</td>
<td>-1.21</td>
<td>-1.20</td>
<td>-1.12</td>
<td>-1.32</td>
<td>-1.22</td>
<td>-0.77</td>
</tr>
<tr>
<td>(R^2)</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
</tr>
</tbody>
</table>

All data were analyzed by using column statistics in GraphPad Prism\textsuperscript{TM}5.0. The data of each carbamate were collected from experiments repeated at least for 4 times.
Initial results obtained from using the DW/OCD method provided values of 50% AChE inhibition rates (IC$_{50}$) (Figure 3-1, Table 3-1). The IC$_{50}$ of these commercial carbamates ranged from 57 nM for recombinant mouse AChE with carbofuran, to 7358 nM for recombinant human AChE with pirimicarb. Carbofuran consistently had the smallest IC$_{50}$ throughout all three vertebrate AChEs. For both ICR mouse brain cortex homogenate and recombinant human AChE, the greatest IC$_{50}$ values were obtained when tested with pirimicarb. For recombinant mouse AChE, the highest IC$_{50}$ was obtained when tested with thiodicarb. The R$^2$ value of all experiments were consistently near 0.99. The value of Hill slope varied from 1.32 (recombinant human AChE + propoxur) to 0.68 (ICR mouse brain cortex homogenate + thiodicarb) and the mean value of it is -1.12. These results indicated that the DW/OCD dilution method was reliable when testing commercial carbamates with the untreated male ICR mouse brain cortex homogenate, recombinant mouse, and recombinant human AChE sample. In addition, AChE activity level and pattern between recombinant mouse AChE and male ICR mouse brain cortex homogenate were well correlated (Figure 3-2). Based on the IC$_{50}$ value tested with the commercial carbamates, a well-fitted linear regression line was obtained and the slope and R$^2$ of the line was 0.99 and 0.96, respectively.
3.2. Solvent Effect on Ellman Assay

However, when I applied the same procedures to the novel carbamate insecticides, I found that all novel carbamates, except PRC391, using the DW/OCD method, did not give results in line with classical enzyme-inhibitor interactions (Figure 3-3). According to the data points from Ellman Assay, as shown above in Figure 3-3a, only 60% of the control AChE activity was reached when the concentration of PRC331 reached 10^{-12} M. In this case, the sigmoidal curve was not accurately formed. Although the Prism\textsuperscript{TM} 5.0 still reported IC\textsubscript{50}, Hill slope and R\textsuperscript{2}, I believed that this data was not reliable. I further diluted the concentration to 10^{-16} M and ran a separate experiment. However, I still failed to fit all data points into one curve (Figure 3-3b). Base on the curve from Figure 18b, the Prism 5.0 cannot obtain a valid IC\textsubscript{50} value and the reported Hill slope was also not reasonable, which was -0.043. In addition, I found that data points formed a plateau from 10^{-7} M to 10^{-12} M and reached 100% at 10^{-14} M.
Figure 3-3. Examples of DW/OCD results of the novel carbamate insecticides. PRC331 gave unusual results for IC₅₀ and Hill slope for Ellman assay, tested on recombinant human AChE (a,b). However, when using dilution with constant DMSO concentration for Ellman assay, an accurate sigmoidal curve was obtained (c).

By using the DW/OCD method, the novel carbamate insecticides would form a thick film on the sodium-phosphate buffer surface in the aliquot after transferred from 0.1 M stock solution. Thus, the suspension in each aliquot was not well formed and the errors would be magnified by further
dilution. So, I switched the dilution method to DWCD (dilution method containing constant 0.1% DMSO), which solved this problem (Figure 3-3c), and was used in all subsequent experiments.

3.3. AChE Inhibition Rate (IC\textsubscript{50}) Testing

All 6 non-target species' AChE enzyme activities were tested by using the Ellman Assay with the DWCD dilution method. Eleven carbamates were tested in this series of experiments, including four commercial carbamates, which were bendiocarb, carbofuran, propoxur and 8 new carbamates, which were PRC331, PRC337, PRC387, PRC388, PRC391, PRC407, PRC408 and PRC421 (Figure 2-1). The results showed that all carbamate insecticides inhibited all six AChE enzymes in a concentration-dependent manner, with varied potencies. Compared to commercial carbamates, novel carbamate insecticides showed moderate AChE inhibition capacity in all non-target species (Table 3-2) and higher selectivity on An. gambiae over non-target species. In the commercial carbamate group, IC\textsubscript{50} ranged from 9 nM for D. magna with carbofuran to 2,094 nM for recombinant human with propoxur. Propoxur in all six species showed the highest IC\textsubscript{50}, ranging from 110 nM for D. magna to 2,094 nM for recombinant humans, while carbofuran in all six species showed the lowest IC\textsubscript{50}, from 9 nM for D. magna to 98 nM for humans. In the novel carbamate group, the IC\textsubscript{50} was overall numerically higher than that of the commercial carbamate group, which ranged from 58 nM for D. magna with PRC337 to 388,800 nM for hen brain homogenate with PRC421. PRC421 in all AChE of the six species showed the highest IC\textsubscript{50}s, ranging from 3,308 nM for D. magna to 388,800 nM for hen brain homogenate, while PRC331 in all six species showed the lowest IC\textsubscript{50}, from 86 nM for D. magna to 373 nM for hen brain homogenate (Table 3-2).

The IC\textsubscript{50} data for An. gambiae were obtained from previous work in the Bloomquist laboratory, which were collected by Daniel Swale, James Mutunga, and Dawn Wong. Based on these data, a selectivity index (IC\textsubscript{50} of all six non-target species / IC\textsubscript{50} of An. gambiae) was
introduced (Table 3-2). The selectivity index in the commercial group remained low in all AChE sources, which ranged from 0.1 for *T. californica* with carbofuran and bendiocarb to 4.0 for humans with propoxur. This number in the novel carbamate insecticide groups varied from 0.3 for *T. californica* with PRC337 to 688 for hen brain homogenate with PRC421 (Table 3-2).
Table 3-2. **IC$_{50}$** and selectivity index data obtained from 6 non-target species acetylcholinesterases using dilution with constant DMSO concentration for Ellman assay dilution.

<table>
<thead>
<tr>
<th></th>
<th>Recombinant Mouse</th>
<th>Recombinant Human</th>
<th>Hen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IC$_{50}$ (nM)</strong></td>
<td>Mean ± S.E.M</td>
<td>Mean ± S.E.M</td>
<td>Mean ± S.E.M</td>
</tr>
<tr>
<td><strong>Selectivity index</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bendiocarb</td>
<td>214 ± 6.4</td>
<td>359 ± 16.2</td>
<td>107.7 ± 18.8</td>
</tr>
<tr>
<td>Carbofuran</td>
<td>60 ± 3.7</td>
<td>97.9 ± 6.9</td>
<td>24.6 ± 2.2</td>
</tr>
<tr>
<td>Propoxur</td>
<td>1,183 ± 73.7</td>
<td>2,094 ± 40.3</td>
<td>317.3 ± 73.3</td>
</tr>
<tr>
<td>PRC 331</td>
<td>171.6 ± 8.8</td>
<td>200.9 ± 18.7</td>
<td>373.2 ± 59.6</td>
</tr>
<tr>
<td>PRC 337</td>
<td>6,863 ± 109.3</td>
<td>10,920 ± 704.2</td>
<td>1,327 ± 233.5</td>
</tr>
<tr>
<td>PRC 387</td>
<td>2,241 ± 336.1</td>
<td>1,079 ± 165.5</td>
<td>6,765 ± 439.2</td>
</tr>
<tr>
<td>PRC 388</td>
<td>264.3 ± 70.2</td>
<td>294.3 ± 21.7</td>
<td>29,430 ± 5,074</td>
</tr>
<tr>
<td>PRC 391</td>
<td>15,830 ± 3,017</td>
<td>1,192 ± 3,952</td>
<td>26,580 ± 6,589</td>
</tr>
<tr>
<td>PRC 407</td>
<td>5,810 ± 1,057</td>
<td>12,070 ± 712.6</td>
<td>6,082 ± 212.3</td>
</tr>
<tr>
<td>RRC 408</td>
<td>22,010 ± 4,781</td>
<td>20,680 ± 9,509</td>
<td>58,760 ± 9,674</td>
</tr>
<tr>
<td>PRC 421</td>
<td>148,400 ± 34,650</td>
<td>247,400 ± 106,800</td>
<td>251,000 ± 60,520</td>
</tr>
</tbody>
</table>

All data showed are in nM range. All data were generated by using column statistics in GraphPad Prism$^{TM}$5.0. Each data set was collected from experiments repeated at least three times.

(Selectivity index = IC$_{50}$ of all six non-target species / IC$_{50}$ of An. gambiae)
<table>
<thead>
<tr>
<th></th>
<th>E. electricus</th>
<th>T. californica</th>
<th>D. magna</th>
<th>An. gambiae (data from previous study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ (nM)</td>
<td>Mean</td>
<td>S.E.M</td>
<td>Selectivity index</td>
<td>Mean</td>
</tr>
<tr>
<td>Bendiocarb</td>
<td>80.6</td>
<td>± 11.7</td>
<td>0.37</td>
<td>11.2</td>
</tr>
<tr>
<td>Carbofuran</td>
<td>20.8</td>
<td>± 2</td>
<td>0.3</td>
<td>9.1</td>
</tr>
<tr>
<td>Propoxur</td>
<td>555.4</td>
<td>± 156.6</td>
<td>1.1</td>
<td>110.8</td>
</tr>
<tr>
<td>PRC 331</td>
<td>113.8</td>
<td>± 17.4</td>
<td>1</td>
<td>85.5</td>
</tr>
<tr>
<td>PRC 337</td>
<td>1,056</td>
<td>± 58.5</td>
<td>6.2</td>
<td>58.3</td>
</tr>
<tr>
<td>PRC 387</td>
<td>335.9</td>
<td>± 23.3</td>
<td>0.9</td>
<td>1,138</td>
</tr>
<tr>
<td>PRC 388</td>
<td>120.9</td>
<td>± 11.7</td>
<td>2.8</td>
<td>1,612</td>
</tr>
<tr>
<td>PRC 391</td>
<td>3,735</td>
<td>± 310.4</td>
<td>34.3</td>
<td>206.1</td>
</tr>
<tr>
<td>PRC 407</td>
<td>1,242</td>
<td>± 40</td>
<td>18</td>
<td>229</td>
</tr>
<tr>
<td>RRC 408</td>
<td>1,241</td>
<td>± 71.8</td>
<td>4.7</td>
<td>240.3</td>
</tr>
<tr>
<td>PRC 421</td>
<td>14,370</td>
<td>± 1,352</td>
<td>39.4</td>
<td>3,308</td>
</tr>
</tbody>
</table>

All data showed are in nM range. Data for each carbamate were repeated at least 4 times. All data were generated by using column statistics in GraphPad Prism™5.0. Each data set was collected from experiments repeated at least three times.

(selectivity index = IC₅₀ of all six non-target species / IC₅₀ of An. gambiae)
Table 3-3. Comparison of the $R^2$-value of the regression line between acetylcholinesterases of different animal species.

<table>
<thead>
<tr>
<th></th>
<th>Recombinant mouse</th>
<th>Recombinant human AChE</th>
<th>Hen brain homogenate</th>
<th>E. electricus</th>
<th>T. californica</th>
<th>D. magna</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant mouse</td>
<td>0.81</td>
<td>0.45</td>
<td>0.93</td>
<td>0.05 *</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>Recombinant human AChE</td>
<td>0.81</td>
<td>0.31</td>
<td>0.70</td>
<td>0.05 *</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>Hen brain homogenate</td>
<td>0.45</td>
<td>0.31</td>
<td>0.40 *</td>
<td>0.55 *</td>
<td>0.32 *</td>
<td></td>
</tr>
<tr>
<td>E. electricus</td>
<td>0.93</td>
<td>0.70</td>
<td>0.40 *</td>
<td>0.05</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>T. californica</td>
<td>0.05 *</td>
<td>0.05 *</td>
<td>0.55 *</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>D. magna</td>
<td>0.81</td>
<td>0.54</td>
<td>0.32 *</td>
<td>0.94</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

$R^2$ value was collected from GraphPad Prism™5.0 as shown in Figure 3-2, following by a paired t-test, to determining any significant IC$_{50}$ value differences among groups in GraphPad Prism™5.0. $P < 0.05$ was considered to be significantly different and asterisk (*) is used to indicate significance.
As mentioned previously, highly correlated IC$_{50}$ value of different carbamate insecticides indicated similarity between two enzymes. Here, the coefficient of determination (R$^2$) was used to determine the activity similarity between two enzymes. Correlations of AChE response pattern and level to the novel carbamates were then compared among six different AChE enzymes by using IC$_{50}$ values obtained from the novel carbamates. The R$^2$ values from the linear regression lines were shown in Table 3-3. The value of R$^2$ varied from 0.05 to 0.94, where E. electricus vs. D. magna had the highest R$^2$ among all pairs. Among all test groups, only 7 of them showed R$^2$ that were above 0.7, which were 0.94 for E. electricus vs. D. magna, 0.93 for E. electricus vs. recombinant mouse, 0.77 for recombinant human vs. recombinant mouse, and 0.70 for E. electricus vs. recombinant human. T. californica had the consistently lowest R$^2$ value when compared to the other species, except when vs. the hen sample (0.55). Paired t-tests showed that there were 5 pairs of enzymes that showed significantly different IC$_{50}$ value, which were all related to T. californica and hen samples. These groups included T. californica vs. recombinant human, T. californica vs. recombinant mouse, T. californica vs. hen, hen vs. E. electricus, and hen vs. D. magna.

3.4. Protein Sequence Alignment

Five AChE protein sequences were obtained for alignment comparison (D. magna AChE protein sequence was not available from NCBI). The summary of the results are presented below (Table 3-4).
Table 3-4. Summary of the multiple sequence alignment using 5 different animal species’ acetylcholinesterases’ protein sequence.

<table>
<thead>
<tr>
<th>Name of sequence</th>
<th>length</th>
<th>vs. Name of sequence</th>
<th>length</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mus</td>
<td>614</td>
<td>Homo</td>
<td>614</td>
<td>88</td>
</tr>
<tr>
<td>Torpedo</td>
<td>596</td>
<td>Electrophorus</td>
<td>633</td>
<td>67</td>
</tr>
<tr>
<td>Mus</td>
<td>614</td>
<td>Electrophorus</td>
<td>633</td>
<td>60</td>
</tr>
<tr>
<td>Homo</td>
<td>614</td>
<td>Gallus</td>
<td>767</td>
<td>60</td>
</tr>
<tr>
<td>Mus</td>
<td>614</td>
<td>Gallus</td>
<td>767</td>
<td>59</td>
</tr>
<tr>
<td>Homo</td>
<td>614</td>
<td>Electrophorus</td>
<td>633</td>
<td>59</td>
</tr>
<tr>
<td>Mus</td>
<td>614</td>
<td>Torpedo</td>
<td>596</td>
<td>58</td>
</tr>
<tr>
<td>Homo</td>
<td>614</td>
<td>Torpedo</td>
<td>596</td>
<td>57</td>
</tr>
<tr>
<td>Gallus</td>
<td>767</td>
<td>Electrophorus</td>
<td>633</td>
<td>55</td>
</tr>
<tr>
<td>Gallus</td>
<td>767</td>
<td>Torpedo</td>
<td>596</td>
<td>54</td>
</tr>
</tbody>
</table>

Length number indicates the number of amino-acids that formed the AChE protein precursor. The score of the alignment number represents the similarity between AChE from different species. The higher the score, the greater genetic similarity they share. All sequence information was obtained from NCBI database (The National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). Mus = mouse, Homo = human, Torpedo = T. californica, Electrophorus = E. electricus, Gallus = hen

For most of the species, AChE precursors were around 610 amino acids (AAs) (Table 3-4). The longest AChE precursor belonged to hen, which was 767 AAs, while the shortest one was T. californica, which was 596 AAs. Human and mouse AChE had the same length which was 614 AAs. At the very right column of Table 4, the scores of pairwise alignment were listed and sorted in a high to low order. I found that highest number was obtained between mouse vs. human, while the lowest number was between hen vs. T. californica. Based on the sequence of the AChE enzyme, a cladogram was obtained and shown below (Figure 3-4):
In Figure 3-4, the cladogram showed that AChE from two mammalian animals, mouse and human, were closely related. Hens were closer to these mammals than the two fish.

3.5. NTE Inhibition Testing by NTE Assay

A measurement of NTE activity inhibition was carried out on hen’s brain homogenate from the lab of Dr. Ehrich Marion. For each single experiment, I used the standard NTE positive control diisopropylphosphorofluoridate (DFP). As illustrated in Table 3-5, DFP (0.5 uM) produced a decreased level of NTE activity, which was 20% of initial NTE activity ($P < 0.0001$, $n = 8$). Then all carbamate insecticides were tested under the same experimental conditions. The data are shown in the Table 3-5. For commercial carbamate insecticides, compared to control group, significant NTE inhibition was observed at 1 mM bendiocarb ($P = 0.0037$, $n = 8$), where the remaining NTE activity was 76% of control. The rest of the commercial carbamate insecticides at 1 mM did not show significant NTE inhibition compared to control NTE reactivity. The remaining NTE activity varied from 93% for PRC337 to 116% for PRC421 (Table 3-5).
Table 3-5. Comparison of neurotoxic esterase assay results by using hen brain homogenate.

<table>
<thead>
<tr>
<th>Hen Sample</th>
<th>% of control</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.00</td>
<td>± 0%</td>
</tr>
<tr>
<td>DFP (0.0005 mM)</td>
<td>19.76 *</td>
<td>± 4.3%</td>
</tr>
<tr>
<td>bendiocarb (1 mM)</td>
<td>76.4 *</td>
<td>± 14%</td>
</tr>
<tr>
<td>carbofuran (1 mM)</td>
<td>100.5</td>
<td>± 9.6%</td>
</tr>
<tr>
<td>propoxur (1 mM)</td>
<td>97.17</td>
<td>± 11.2%</td>
</tr>
<tr>
<td>PRC331 (1 mM)</td>
<td>101.1</td>
<td>± 11.8%</td>
</tr>
<tr>
<td>PRC337 (1 mM)</td>
<td>93.32</td>
<td>± 12.8%</td>
</tr>
<tr>
<td>PRC387 (1 mM)</td>
<td>111.5</td>
<td>± 17.6%</td>
</tr>
<tr>
<td>PRC388 (1 mM)</td>
<td>98.59</td>
<td>± 11.7%</td>
</tr>
<tr>
<td>PRC391 (1 mM)</td>
<td>97.41</td>
<td>± 12.7%</td>
</tr>
<tr>
<td>PRC407 (1 mM)</td>
<td>101.5</td>
<td>± 12.8%</td>
</tr>
<tr>
<td>PRC408 (1 mM)</td>
<td>110.9</td>
<td>± 16.9%</td>
</tr>
<tr>
<td>PRC421 (1 mM)</td>
<td>116.4</td>
<td>± 26.1%</td>
</tr>
</tbody>
</table>

Amount of remaining NTE activity of hen brain homogenate tested on both commercial and novel carbamate insecticides. Each result was the average of experiments repeated at least 3 times. The one-way ANOVA with Dunnett’s post test was conducted to detect difference from control in GraphPad Prism™ 5.0. Asterisk (*) represents significant difference.
3.6. *In vivo* *D. magna* Aquatic Toxicity Bioassay

In this set of experiments, concentrations as high as $10^{-5}$ M led to 100% mortality in *D. magna* for all carbamate insecticides. Bendiocarb was used as a representative of commercial carbamates. The sigmoidal curve analysis showed that its LC$_{50}$ (median lethal concentration) was 611 nM (Table 3-6). In the novel carbamate group, 7 of them presented LC$_{50}$ values at about the same potency as bendiocarb, which were PRC337, PRC 387, PRC388, PRC391, PRC407, PRC408 and PRC421. Among these seven novel carbamates, PRC407 and PRC408 had the lowest LC$_{50}$, while PRC421 had the highest LC$_{50}$. PRC331 was the only novel carbamate that presented significantly lowered (3.5-fold) LC$_{50}$ value compared to bendiocarb ($P = 0.0349$).

Table 3-6. *In vitro* toxicity aquatic bioassay results by using *D. magna*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LC$_{50}$ (nM)</th>
<th>S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td>bendiocarb</td>
<td>611</td>
<td>± 179</td>
</tr>
<tr>
<td>PRC331</td>
<td>172 *</td>
<td>± 28</td>
</tr>
<tr>
<td>PRC337</td>
<td>439</td>
<td>± 236</td>
</tr>
<tr>
<td>PRC387</td>
<td>1109</td>
<td>± 549</td>
</tr>
<tr>
<td>PRC388</td>
<td>258</td>
<td>± 130</td>
</tr>
<tr>
<td>PRC391</td>
<td>755</td>
<td>± 243</td>
</tr>
<tr>
<td>PRC407</td>
<td>225</td>
<td>± 56</td>
</tr>
<tr>
<td>PRC408</td>
<td>225</td>
<td>± 62</td>
</tr>
<tr>
<td>PRC421</td>
<td>834</td>
<td>± 63</td>
</tr>
</tbody>
</table>

Each data number was the average of experiment results repeated at least for 3 times. One-way ANOVA with Dunnett’s post test was conducted to detect difference from the standard in GraphPad Prism™5.0. Asterisk (*) represents significant difference between bendiocarb and experimental compound.
Initial studies established experimental conditions to be used throughout this research. Data from these experiments using DWOC (Table 3-1, Figure 3-1) were similar to previous studies. It is reported that the IC$_{50}$ for aldicarb was 2543 nM (postnatal day 4 rat, incubation time 30 min), for carbofuran was 150 nM (8 weeks old rat plasma cholinesterase, incubation time 1 min) and for carbaryl was 1758 nM (postnatal day 4 rat, incubation time 30 min) (Lyaniwura 1989; Mortensen et al. 1998). Thus, results from rat were close to the mouse IC$_{50}$ values that I measured. As expected, there was also a close correlation between recombinant murine AChE and that found in brain cortex homogenates, which suggests that the recombinant enzyme faithfully reflects that of the native, at least from the perspective of carbamate action.

Subsequent studies explored the effects of DMSO as a solvent compound, for which it is widely used in both chemical and biological research (Tjernberg et al. 2006). Its chemical properties allow it to be soluble in both organic and non-organic media because of the combination of one polar domain and two non-polar domains (Santos et al. 2003). As a hydrogen-bond disruptor (Santos et al. 1997), DMSO has been shown to affect normal cell physiological process, such as cell cycle adjustment, differentiation and apoptosis (Parkin et al. 1997). When the commercial carbamates were evaluated, all data fitted well to a sigmoidal curve and I obtained reasonable $R^2$ and Hill slope values. However, when applying the same procedure to the novel carbamates, the poor solubility prevented them from forming a well-distributed suspension. A film was formed on the top of the sodium-phosphate buffer when I transferred 100 ul 0.1 M stock solution into the aliquot. Even extensive vortexing would not
distribute the compound into the buffer. Subsequent dilution further amplified this error, giving spurious results.

Then I switched the dilution method from DW/OCD to DWCD. The results showed that the commercial carbamates had a low IC$_{50}$ and potent inhibition capacity on AChE, in vitro, on non-target animal species. In addition, I showed that bendiocarb, carbofuran, and propoxur were poorly selective on AChE of non-target species. As mentioned before, the potent AChE inhibition capacity and low selectivity of commercial carbamates have long been a serious safety issue for mammals, livestock, and the aquatic environment, especially in areas relying on carbamates for malaria control and agriculture. For the novel carbamates, AgAChE inhibition capacity was comparable to that of the commercial carbamates (Swale 2009; works from Dawn Wong and James Mutunga). However, their selectivity indices were much higher than the commercial carbamates, indicating less AChE inhibition potency on non-target species. Thus, this set of data suggests a more prominent safety property of the novel carbamates. Because of the complexity of pharmacokinetic processes, in vitro bioassay cannot give an exact prediction of in vivo toxicity. Thus, in future work, in vivo bioassay using small vertebrate animals should be conducted. I would expect to see less acute lethality on those non-target species in vivo, which would confirm the safety properties of the novel carbamates obtained, in vitro.

This study also offered me the insight into the structure-activity relation of carbamates and the preferred structures for different AChEs. The branched chain on the phenyl ring affects the AChE inhibition potency of carbamates greatly (Kuhr and Dorough 1976). Generally speaking, alkyl substituents at the meta- position make a carbamate a more potent enzyme inhibitor than at the ortho- position (Kuhr and Dorough 1976). This statement is supported by my results, since the ortho-substituted carbamates on the phenyl ring have a higher IC$_{50}$ than
meta- position carbamates. However, meta-substituted carbamates are not as potent AChE inhibitors as carbofuran or bendiocarb (essentially benzofurans with 1 [carbofuran] or 2 [bendiocarb] oxygens, the latter known as dioxolane). The benzofuran/dioxolane materials are also the least selective, probably because their structures are more rigid, thereby optimizing interaction with highly conserved substructures of the CAS across species. Conversely, they lack the side chain flexibility that might accommodate various substructures and protein conformations of different species, and contribute to selectivity. In conclusion, the benzofuran and similar structures are not good options for selective carbamate insecticide design.

In my study, I used propoxur, PRC 337, PRC391, PRC407, PRC408 and PRC421, which areortho-substituted carbamates (orthoposition side chain on the phenyl ring). Propoxur and PRC421 are two ortho-substituted carbamates that use oxygen to link the phenyl ring and the side chain while PRC337, PRC391, PRC407 and PRC408 use the sulfur (Table 2-1). For all non-target species, propoxur usually has the lowest IC\textsubscript{50} while the PRC421 had the greatest (Table 3-2). As mentioned above, increasing side chain length from 1 carbon to 3 carbons could enhance the anticholinesterase capacity, but decreases it when the side chain is longer than 4 carbons (Kuhr and Dorough 1976). Comparing the chemical structures, PRC421 has a longer (1 oxygen and 4 carbons side chain backbone) and more branched side chain than propoxur (1 oxygen and 2 carbons side chain backbone). The co-effect of these two factors makes PRC421 have a much higher IC\textsubscript{50} value than propoxur throughout all six species. Although PRC421 also decreased AgAChE inhibition potency, the severe effect on non-target AChE inhibition potency increased the selectivity index throughout all species tested. This finding offers a promising structure for selective carbamate insecticide design.
In the novel carbamate group, there are three meta-substituted carbamates, which are PRC331, PRC387 and PRC388 (Table 2-1). PRC331 is the only compound that uses carbon to link the side chain and phenyl ring. Compared to the other two carbamates, its IC$_{50}$ value was also the smallest throughout all six species’ AChE (Table 3-1). However, when the carbon connecting the phenyl ring and alkyl side chains is replaced with silicon (PRC387), the IC$_{50}$ value of PRC387 increased from 2.95-fold for *E. electricus* (from 113.8 nM to 335.9 nM) to 18-fold for hen (from 373.2nM to 6,765 nM). Apparently, the slightly longer carbon-silicon bond, which is 1.89 Å compared to 1.54 Å for carbon-carbon bond (Allen et al. 1987) can induce an apparent IC$_{50}$ shift. This finding suggests a narrow site in the all non-target species’ AChE, which fits carbon-carbon bond better than carbon-silicon bond. Comparing one side chain that is ethyl (PRC387) to methyl (PRC388), increased the AChE inhibition potency in most species, but not hen (changed from 6,765 nM to 29,430 nM) and *T. californica* (changed from 1,138 nM to 1,612 nM). For the hen and *T. californica*, the 4.3-fold and 1.4-fold IC$_{50}$ value increase suggest that the ethyl side chain is too long. For the rest of the non-target species, ethyl groups fit better in this position on their AChE. However, potency on AgAChE showed an even greater potency increase from this structural change (8.7-fold potency increase), which had an overall effect of prominently increasing the selectivity index across all non-target species.

AChE structure and sensitivity to inhibitors varies between different species. The two mammalian AChEs, which were recombinant human AChE and recombinant mouse AChE, appeared to have a similar sensitivity pattern and level to the novel carbamates (Table 3-3) and highly similar protein sequence alignment (Table 3-4). In addition, the cladogram tree showed they were evolutionarily more closed to each other than any other tested species. Hen AChE was genetically and evolutionarily not very close to the human and mouse, as indicated by the
moderate score from the AChE protein sequence alignment and far taxa position on the cladogram. My experimental data verified this relationship by showing a different sensitivity pattern to the novel carbamates ($R^2<0.45$) between the hen and other two mammalian species. However, based on my findings, hen AChE was more closely related to mammalian AChE than that of the aquatic species. In aquatic groups, *E. electricus* vs. *D. magna* had the highest $R^2$ (0.94) and similar carbamate sensitivity levels. Thus, although the AChE protein sequence of *D. magna* is currently not available online, it would be reasonable to hypothesize that AChE of *D. magna* would be more genetically and evolutionarily close to that of *E. electricus*. It was very surprising to see that *E. electricus* and *T. californica* had a different AChE sensitivity pattern to the novel carbamates ($R^2 = 0.05$). According the data, they shared the second highest score of AChE protein sequence alignment (66) and similar AChE sensitivity level to the novel carbamates. Apparently, their structures differ in accommodating novel carbamate side chains in a way not obvious from their protein sequence.

NTE inhibitors are categorized into two groups, which are neuropathic and non-neuropathic NTE inhibitors (Glynn 2006; Moretto and Lotti 2006). Aging only occurs in the NTE-neuropathic organophosphate inhibitor complex, which causes OPIDN (Glynn 2006). However, because of the finding that some carbamates are capable of inducing polyneuropathy at high doses (Lotti and Moretto 2006; Randall et al. 1997), the safety testing for carbamates now also includes NTE toxicity testing. In my study, I used hen brain homogenate to test the NTE inhibition on both commercial and novel carbamate insecticides. The results showed that not all commercial carbamate insecticides but all novel commercial carbamate insecticides have no adverse effect on NTE. The exception was bendiocarb, which at 1 mM could inhibit NTE *in vitro*. Because there is no literature demonstrating whether bendiocarb can cause OPIDN, it will
be very interesting to take a further look at, not only bendiocarb, but other commercial carbamates. Overall, this set of experiments suggested that the novel carbamates are unlikely to cause delayed neuropathy via inhibition of NTE. In future work, in vivo bioassay using hen should be conducted to confirm this speculation. The safety features of the novel carbamates will be reconfirmed if they are not OPIDN-inducing in this model.

*D. magna* is recommended by the Food and Drug Administration (FDA) and Office of Pollution Prevention and Toxics (OPPT) as a standard animal model for acute or chronic toxicity testing (Versteeg et al. 1997), and has been regarded as one of the most reliable indicators for exposing potential environmental problems (Dodson and Hanazato 1995). Thus, I used *D. magna* as an in vivo non-target animal model. In addition, because bendiocarb has been well studied in aquatic toxicology (PAN Pesticides Database 2000), bendiocarb was selected as representative of the commercial carbamates. According to the literature, the 24-hour acute toxicity for bendiocarb is 1.4 uM in in vivo aquatic assay using *D. magna* (PAN Pesticides Database 2005). As compared to the LC$_{50}$ data (0.61 uM) I obtained, it is convincing that my bioassay system is accurate. PRC 331 is the only novel carbamate that showed significantly lowered LC$_{50}$ compared to bendiocarb, with the rest of the novel carbamates having similar killing potency as bendiocarb. Bendiocarb has been categorized as a carbamate with moderate to high toxicity to aquatic animal species, including both the vertebrate (fish) and the invertebrates (shrimp, crabs, and insects) (PAN Pesticides Database 2000; Wright et al. 1981). In this case, because of its more potent aquatic toxicity to non-target species than the existing commercial carbamates (bendiocarb), PRC331 is not a good option for *An. gambiae* larva control for aquatic application. However, since it is envisioned to use this compound in bednets or as IRS, I think it is still a good candidate for *An. gambiae* control in other life stages, such as adult stage. For the
rest of the novel carbamates, which are PRC337, PRC387, PRC388, PRC391, PRC407, PRC408 and PRC421, further *An. gambiae* larvae *in vivo* bioassay will need to be conducted. If the data could prove that they have a lower LC$_{50}$ and higher killing capacity on *An. gambiae* larvae compared to the commercial carbamates, they might be developed as optimum candidates for both *An. gambiae* adult and larva control.

### Chapter 5 Conclusions

For malaria control, the carbamate insecticides presently in use (propoxur and bendiocarb) are mostly non-selective, and inhibit both target (mosquito) AChE and non-target (human and other mammalian) AChE. Because these non-selective carbamate insecticides have been used in malaria endemic areas, safety concerns are raised because of intoxication of humans and other non-target species. Thus, it is reasonable to develop some selective carbamate insecticides, which would selectively inhibit target AChE but not the others. Overall, I obtained convincing results that, *in vitro*, the novel carbamate insecticides have improved properties compared to the WHO Pesticide Evaluation Scheme (WHOPES) standards propoxur and bendiocarb.

In this study, I first showed the accuracy of my testing system by evaluating nine commercial carbamates on three vertebrate AChE enzyme sources in the Ellman assay, which used a DW/OCD method for preparation. However, because DW/OCD was not shown to be reliable for the novel carbamates, I switched to DWCD method instead and obtained good results.

After selecting the optimum preparation method, I started collecting IC$_{50}$ values and found selectivity differences between the commercial carbamates and the novel carbamates. Compared to
the commercial ones, I found that the novel carbamates had a higher selectivity on target AChE species (*An. gambiae*). In addition, NTE assay was conducted and the data showed that the novel carbamates were not toxic to NTE at 1 mM concentration. These two sets of experiment provided information that the novel carbamates have a strong inhibition and high selectivity for AChE of target species and should not be neuropathy-inducing.

AChE activity and protein sequence were compared throughout all non-target species. I found that mouse and human AChE were highly similar in sensitivity pattern and level to the novel carbamates, AChE protein sequence, and evolutionary relationship. For the two aquatic fishes, although they also have a moderate alignment score and similar AChE activity, they had a different patterns of response to the novel carbamates. Hen has a higher alignment score with the two mammalian organisms than that of the aquatic animals. However, it had a different reaction pattern to the novel carbamates compared to the mouse and human. Thus, I conclude that mouse and human AChE are most similar to each other both genetically, evolutionarily, and functionally. Hen AChE is genetically more close to the mammalian but has a unique AChE activity pattern to the novel carbamates.

For *in vivo* aquatic toxicology bioassay using *D. magna*, PRC331 showed a significantly lowered LC$_{50}$ compared to bendiocarb, which suggested that it is not a good candidate for *An. gambiae* larvae control. The safety features of PRC337, PRC387, PRC388, PRC391, PRC407, PRC408 and PRC421 allow them to be considered as options for both *An. gambiae* larvae and adult control.

For future study, *in vivo* bioassay using *An. gambiae* and small vertebrate animals should be conducted to confirm the safety property of the novel carbamates in real conditions. All novel carbamates should also be tested in aquatic toxicology assay on *An. gambiae* larvae stages.
the contact, fumigant, and repellent bioassay should also be done on adult *An. gambiae* with all novel carbamates.
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