Inhibitor profile of bis(n)-tacrine and N-methylcarbamates on acetylcholinesterase from Rhipicephalus (Boophilus) microplus and Phlebotomus papatasi

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ABSTRACT

The cattle tick, Rhipicephalus (Boophilus) microplus (Bm), and the sand fly, Phlebotomus papatasi (Pp), are disease vectors to cattle and humans, respectively. The purpose of this study was to characterize the inhibitor profile of acetylcholinesterases from Bm (BmAChE1) and Pp (PpAChE) compared to human and bovine AChE, in order to identify divergent pharmacology that might lead to selective inhibitors. Results indicate that BmAChE has low sensitivity (IC50 = 200 µM) toward tacrine, a monocovalent catalytic site inhibitor with sub micromolar blocking potency in all previous species tested. Similarly, a series of bis(n)-tacrine dimer series, bivalent inhibitors and peripheral site AChE inhibitors possess poor potency toward BmAChE. Molecular homology models suggest the rBmAChE enzyme possesses a W384F orthologous substitution near the catalytic site, where the larger tryptophan side chain obstructs the access of larger ligands to the active site, but functional analysis of this mutation suggests it only partially explains the low sensitivity to tacrine. In addition, BmAChE1 and PpAChE have low nanomolar sensitivity to some experimental carbamates anticholinesterases originally designed for control of the malaria mosquito, Anopheles gambiense. One experimental compound, 2-(((2-ethylbutyl)thio)phenyl)methylcarbamate, possesses >300-fold selectivity for BmAChE1 and PpAChE over human AChE, and a mouse oral LD50 of >1500 mg/kg, thus providing an excellent new lead for vector control.

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1. Introduction

Utilization of insecticides for disease vector control remains the most effective component of the integrated vector management approach for control of vector borne diseases [1]. The cattle tick, Rhipicephalus (Boophilus) microplus (Canestrini; Bm), is a poten-
**Inhibitor profile of bis(n)-tacrines and N-methylcarbamates on acetylcholinesterase from Rhipicephalus (Boophilus) microplus and Phlebotomus papatasi**

The cattle tick, *Rhipicephalus (Boophilus) microplus* (Bm), and the sand fly, *Phlebotomus papatasi* (Pp), are disease vectors to cattle and humans, respectively. The purpose of this study was to characterize the inhibitor profile of acetylcholinesterases from Bm (BmAChE1) and Pp (PpAChE) compared to human and bovine AChE, in order to identify divergent pharmacology that might lead to selective inhibitors. Results indicate that BmAChE has low sensitivity (IC50 = 200 μM) toward tacrine, a monovalent catalytic site inhibitor with sub micromolar blocking potency in all previous species tested. Similarly, a series of bis(n)-tacrine dimer series, bivalent inhibitors and peripheral site AChE inhibitors possess poor potency toward BmAChE. Molecular homology models suggest the rBmAChE enzyme possesses a W384F orthologous substitution near the catalytic site, where the larger tryptophan side chain obstructs the access of larger ligands to the active site, but functional analysis of this mutation suggests it only partially explains the low sensitivity to tacrine. In addition, BmAChE1 and PpAChE have low nanomolar sensitivity to some experimental carbamate anticholinesterases originally designed for control of the malaria mosquito *Anopheles gambiae*. One experimental compound, 2-((2-ethylbutyl)thio)phenyl methylcarbamate, possesses >300-fold selectivity for BmAChE1 and PpAChE over human AChE, and a mouse oral LD50 of >1500 mg/kg, thus providing an excellent new lead for vector control.
Although these control methods have been effective in reducing Boophilus and Phlebotomus populations, control has become increasingly difficult due to escalating insecticide resistance among wild populations [5,12–14]. OP pesticides, such as coumaphos, are inhibitors of AChE (EC 3.1.1.7), a serine hydrolase responsible for terminating nerve signals at the synapses of cholinergic systems within the central nervous system of invertebrates, leading to death [15]. OP and pyrethroid resistance has been attributed to both metabolic and target site mechanisms, with the latter being the primary reason for OP resistance [12,16–19]. OP-insensitive AChE might provide cross resistance to insecticides with similar mode of action, such as carbamates. Modification of current compounds can provide increased invertebrate/vertebrate selectivity ratios alongside the potential for development of resistance-mitigating compounds.

The three-dimensional crystal structures of AChE from Tc [20], Dm [21], and mouse [22] (among others) are available, and provide insights on structure–function relationships for numerous inhibitors. Pharmacological and structural analyses of AChE have revealed that AChE contains two binding sites for inhibitors: one at the CS and one near the entrance to the catalytic gorge, the PS [20–22]. The CS is located about 4 Å from the base of the gorge and is defined (in part) by the catalytic triad S200, H440, E327, as well as W84 (Tc numbering), the latter serving to bind the trimethylammonium group of acetylcholine [23]. In turn, the PS is located toward the mouth of the gorge and consists of W279, Y70, D72, and Y121 (Tc numbering) [24–27]. The PS has been shown to briefly bind substrates en route to the CS, thereby increasing catalytic efficiency [24,25]. Using differences in CS geometry between AgAChE and hAChE, we have developed anticholinesterase mosquitocides (carbamates) having mosquito selectivity of up to 500-fold [28]. Simultaneous occupancy of the CS and PS sites through the design of bivalent inhibitors should facilitate the mitigation of AChE target site resistance, since resistance to this type of compound would require the development of multiple mutations in the protein while retaining sufficient functionality.

In this study, we characterized the inhibitor profile of acetylcholinesterases from rBmAChE1 and PpAChE compared to human and bovine AChE, in order to identify divergent pharmacology that might lead to selective inhibitors. Secondly, we show evidence of highly potent and selective experimental carbamate inhibitors that can assist in the control of Bm and Pp populations.

2. Methods

2.1. Inhibitors, solvents, and assay reagents

Propoxur (purity ≥ 99%), bendiocarb (purity ≥ 99%), edrophonium (purity ≥ 98%), eserine (purity ≥ 99%), and tacrine (purity ≥ 99%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Experimental carbamates (Fig. 1) were prepared as described in Carlier et al. [28]. All experimental compounds were purified by column chromatography and/or re-crystallization, and were >95% pure by 1H NMR analysis. Carbamate and tacrine-based inhibitors used in this study are shown in Fig. 1. Bis(n)-tacrine dimers (n = 2, 3, 4, 5, 6, 7, 8, 9, 10, and 12 methylenes) were synthesized and purified to >95% using established procedures [29]. The inhibitors donepezil (≥98% purity), BW284c51 (≥98% purity), tubocurarine (≥97% purity), and ethidium (95% purity) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Ellman assay [30] reagents are composed of ATCh (≥99% purity), DTNB (99% purity), and sodium phosphate buffer, all of which were purchased from Sigma–Aldrich (St. Louis, MO, USA). Molecular sieve OP type 3 Å beads were purchased from Sigma (St. Louis, MO, USA) and were used to prevent water absorption within the DMSO stock.

2.2. Molecular homology modeling

Homology model of the BmAChE1 was constructed in ICM [31]. X-ray structure from the Protein Data Bank (PDB ID 1ACJ – the complex of Torpedo California AChE with tacrine) was used as a template. Side-chain refinement was performed in ICM using a Biased Probability Monte–Carlo (BPMC) global optimization procedure [32].

2.3. Enzyme preparations

Recombinant constructs of R. (B.) microplus BmAChE1 (Table 1) were produced as previously described [33], except that baculovirus supernatants containing rBmAChE1 were produced in sf21 insect cell culture grown in Gibco® SF-900™ III SFM (serum-free medium, Life Technologies, Carlsbad, CA). Site-directed mutagenesis was utilized to convert the codon for W384 to F384 (W384F) in BmAChE1 (Deutch #5, wt) pre-cloned into the baculoviral transfer plasmid pBlueBac4.5/B5-His-TOPO® (Life Technologies) as previously described [35]. Briefly, 5’-phosphorylated PCR primers BmAChE1-1203U29X (CTTCTTCTTGCAATACTTCTTCGGATTTC) and BmAChE1-1181L22 (GAACCTTCGTTTGGCTTAGAAC) were utilized to 25 cycles, 66 °C annealing temp, 4 min extension at 72 °C) with the Phusion® Site-Directed Mutagenesis Kit (Thermo Fisher Scientific, Pittsburg, PA) to perform targeted mutagenesis following the instructions of the manufacturer. The mutated plasmid was transformed into Escherichia coli TOP10 chemically competent cells, sequence verified, and co-transfected with Bac-N-Blue™ DNA into SF21 insect cells as previously described [35]. Baculovirus cultures were produced in sf21 cells grown in Gibco SF-900™ III SFM. Baculoviral DNA was isolated and sequenced from all expression cultures to verify construction and expression of the intended coding sequences.

Six enzymes were utilized in this study: rBmAChE1 and mutated rBmAChE1 (W384F), prepared as described above, rPpAChE, hAChE, bovine brain homogenate, and AgAChE homogenate. AgAChE and bovine brain homogenate enzyme was prepared from groups of ten whole non-blood fed adult female mosquitoes or

![Fig. 1. Structure of bis(n)-tacrines, where n = 2, 3, 4, 5, 6, 7, 8, 9, 10, and 12 methylenes, as well as the experimental methylcarbamate inhibitors used in this study.](image-url)
5 mg (wet weight) of excised bovine brain tissue. Bovine tissue collection was via a local slaughterhouse, and approved by the University of Florida IACUC. Each tissue was homogenized in 1 mL of ice-cold sodium phosphate buffer (0.1 M, pH 7.8) containing 0.3% Triton x-100, with an electric motor driven glass tissue homogenizer. The homogenate was centrifuged at 5000 g for 5 min. The supernatant was used as the enzyme source for the assay. Prior to use, \( \text{BmAChE1} \) and \( \text{rPpAChE} \) were diluted 10\(^{-4}\) and \( \text{hAChE} \) was diluted 100\(^{-4}\) with the aforementioned buffer + Triton mixture.

### 2.4. Enzyme inhibition assays

\( \text{IC}_{50} \) values were determined using slight modifications from Ellman et al. [30], as outlined in Hartsel et al. [28]. Briefly, 10 \( \mu \)L of enzyme solution was added to each well of a 96-well microtiter plate, along with 20 \( \mu \)L of dissolved compound and 150 \( \mu \)L of ice-cold phosphate buffer. The assay plate was incubated at 25 °C for 10 min. Ellman assay reagents, ATCh (0.4 mM, final concentration) and DTNB (0.3 mM, final concentration) were prepared fresh, and 20 \( \mu \)L was added to the enzyme to initiate the reaction. Changes in absorbance were recorded by a DYNeX Triad spectrophotometer (DYNeX Technologies, Chantilly, VA, USA) at 405 nm. Six inhibitor concentrations were used in triplicate to construct concentration–response curves. Inhibitors were dissolved in DMSO and stocks were diluted to give a final concentration of 0.1% DMSO (v/v) in the microtiter plate for each inhibitor concentration. Enzyme concentrations used were within the linear range of activity, therefore eliminating the need for protein quantification.

### 2.5. Mammalian toxicity studies

We performed a toxicity screen of three experimental carbamate insecticides 1, 6, and 7 using the OECD approved method, known as the “up-and-down method” [36], for oral dosing of male Mus musculus (ICR strain). The University of Florida IACUC approved all procedures for these experiments. Standard oral treatments used olive oil mixtures (≤400 \( \mu \)L) administered through a gavage needle. A maximum of eight mice total were used for each inhibitor and were monitored every 4 h for 24 h after the administration of the insecticide. Toxicity was recorded at 24 h post exposure. The mice were sacrificed at any sign of suffering and counted as dead.

### 2.6. Statistical analyses

Individual \( \text{IC}_{50} \) values were calculated using nonlinear regression with GraphPad Prism™ (GraphPad Software, San Diego, CA, USA). All experiments yielded acceptable Hill slope (>0.8) and \( r^2 \) (>0.99) values. \( \text{IC}_{50} \) values are expressed as mean of \( n = 3 \) values. Mean \( \text{IC}_{50} \) values and 95% confidence limits were determined with GraphPad InStat™ (GraphPad Software, San Diego, CA, USA). The mean \( \text{IC}_{50} \) values were statistically analyzed using an unpaired \( t \)-test (two tail) and Tukey’s post-test with significance being represented by \( P < 0.05 \). Statistical analyses were performed using InStat™ (GraphPad Software, San Diego, CA, USA). Selectivity ratios of enzymes were determined by the equation: vertebrate \( \text{IC}_{50} \) / invertebrate \( \text{IC}_{50} \). Estimates of murine LD\(_{50} \) values were calculated in the SAS statistical program (Cary, NC).

### 3. Results

#### 3.1. Potency of \( \text{AChE} \) inhibitors in arthropods

The majority of compounds used for enzyme characterization act at the CS of \( \text{AChE} \). Coumaphos oxon is a potent anticholinesterase against \( \text{BmAChE1} \) (Table 2), but is 43-fold less effective against \( \text{rPpAChE} \) (Table 2). Commercial carbamate insecticides (propoxur, carbaryl, and bendiocarb) are highly potent inhibitors of both \( \text{BmAChE1} \) and \( \text{rPpAChE} \) (Table 2). For both \( \text{rBmAChE1} \) and \( \text{rPpAChE} \),

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* Amino acid substitutions compared to GenBank accession CAAC11702 are indicated below each recombinant construct expressed in the baculovirus system. Bold underline type indicates substitutions present only in constructs described in more detail elsewhere [34], along with sensitivity to coumaphos-oxon (Table 5).
the most active carbamate was carbofuran, whereas in the tick propoxur was the least active, and for the sandfly it was carbayl. Although commercial carbamates yielded fairly similar inhibition potencies for both \( \text{rPpAChE} \) and \( \text{rbmAChE} \), experimental carbamates possessed varying potencies against the two enzymes (Table 2). For \( \text{rbmAChE} \), experimental carbamates possess an activity range of approximately 25-fold, with 6 and 3 being the most and least potent inhibitor, respectively. Compound 1 is the most potent experimental carbamate with a \( \text{meta} \) positioned side chain by approximately 5- to 10-fold, compared to 2 and 3, respectively. Compound 6 displayed the greatest potency for experimental inhibitors studied with an \( \text{ortho} \) positioned side chain. For \( \text{rPpAChE} \), experimental inhibitors displayed a range of 18-fold with 1 and 7 being the most and least potent inhibitors, respectively (Table 2). The \( \text{meta} \)-substituted compounds were of similar high potency, with compound 6 the only \( \text{ortho} \) carbamate of similar activity. This compound was also equipotent against both \( \text{rbmAChE} \) and \( \text{rPpAChE} \). \( \text{rbmAChE} \) was found to have low sensitivity (IC\(_{50} = 221 \text{ µM} \)) toward tacrine, a monovalent CS inhibitor with sub micromolar blocking potency in all previous species tested, including \( \text{rPpAChE} \). Eserine, a natural product CS-directed inhibitor was the most potent compound tested in this study, and its activity favors \( \text{rbmAChE} \) over \( \text{rPpAChE} \) by a factor of six (Table 2). Edrophonium, a reversible CS inhibitor, was considerably less potent than the carbamates, and displayed similar mean IC\(_{50} \) values in the low micromolar range against \( \text{rbmAChE} \) and \( \text{rPpAChE} \) (Table 2).

The two peripheral site inhibitors studied (tubocurarine and ethidium) displayed poor inhibition of \( \text{rbmAChE} \), but typical levels of inhibitory activity to \( \text{rPpAChE} \). Tubocurarine inhibited no more than 15% of enzyme activity at 1 \( \text{µM} \) with \( \text{rbmAChE} \), and was roughly 25-fold more active against \( \text{rPpAChE} \) (Table 2). Ethidium was more active than tubocurarine in both species, and showed 5-fold greater potency for \( \text{rPpAChE} \) than \( \text{rbmAChE} \).

Bivalent AChE inhibitors spanning both the CS and PS binding domains possessed reduced inhibition potency toward \( \text{rbmAChE} \) and high potency toward \( \text{rPpAChE} \). BW284C51 was active at the low micromolar level on \( \text{rbmAChE} \), and was found to have 424-fold greater activity against \( \text{rPpAChE} \) (Table 2). Donepezil showed approximately three percent inhibition at 100 \( \text{µM} \), and was therefore considered to be inactive on \( \text{rbmAChE} \). However, Donepezil was a potent inhibitor of \( \text{rPpAChE} \), as it was found to have an IC\(_{50} \) value of ca. 100 nM (Table 2). To further understand the inhibitor profile of \( \text{rbmAChE} \) and \( \text{rPpAChE} \), the enzymes were also studied using a bis(-)-tacrine dimer series as structural probes to measure the distance between the CS and PS (Table 3). \( \text{rPpAChE} \) was found to be more sensitive to the entire tacrine dimer series when compared to \( \text{rbmAChE} \). Comparing IC\(_{50} \) values across \( \text{rbmAChE} \) and \( \text{rPpAChE} \), the differences in potency ranged from 297-fold for bis(8)-tacrine to 1493-fold for bis(10)-tacrine (Table 3). For \( \text{rbmAChE} \), the most potent tacrine dimer was found to be bis(8)-tacrine and the least potent was found to be bis(2)-tacrine. However, a different pattern of inhibition for \( \text{rPpAChE} \) produced less than a 2.5-fold difference between bis(7)-tacrine through bis(12)-tacrine, with the IC\(_{50} \) values ranging from 2–5 nM (Table 3).

### 3.2. Potency of AChE inhibitors in mammals

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

Mammalian enzymes were also found to possess similar sensitivities to both peripheral site inhibitors studied (Table 2). The potency differences between the two species was 1.05-fold and 1.4-fold for tubocurarine and etidium, respectfully. Etidium was found to be more potent than tubocurarine to both enzymes by approximately 2-fold.

Bivalent inhibitors, donepezil and BW284c51, were shown to be the most potent blockers of both mammalian enzymes, with IC

50 values in the 7–33 nM range (Table 2). Donepezil was the most active inhibitor of both mammalian enzymes, with nearly equipotent IC

50 values against hAChE bovine AChE. Although 2- to 4-fold less potent than donepezil, BW284c51 was still the second most potent inhibitor against hAChE and bovine AChE, with inhibition values differing by 2.1-fold across these species.

3.3. Inhibitor selectivity across mammals and arthropods

SR values are used to express in vitro selectivity differences between mammalian and arthropod enzymes, as shown in Table 2. For rBmAChE1, the most selective standard carbamate was carbaaryl when compared to both human and bovine enzymes. Otherwise, commercial carbarbates were shown to have a range of enzyme selectivity that varied from 6- to 174-fold for hAChE and 12- to 163-fold for bovine AChE. Edrophonium was found to be negatively selective for both the human and bovine AChE enzymes, as they were more active on the mammalian enzyme when compared to rBmAChE1. Eserine was found to be highly selective for rBmAChE1 over bovine AChE (123-fold), and moderately selective over hAChE (46-fold). The SR of commercial carbarbates for rBmAChE1 were shown to be highly variable and ranged from 1-fold to 360-fold for hAChE and 4-fold to 338-fold for bovine AChE (Table 2). The most potent experimental inhibitor, 6, presented the largest SR (338-fold) for bovine AChE and was also found to be very selective against hAChE with an SR of 342. The lowest SR observed for rBmAChE1 and bovine hAChE was 3, with SR values of 4-fold and 1-fold, respectively. Coumaphos oxon was found to have SR of 104-fold and 11-fold for bovine and hAChE, respectively. The SR values for 6 are 3.3-fold (bovine AChE) and 31-fold (hAChE) better than the currently used acaricide, coumaphos oxon. PS and bivalent inhibitors were both found to be negatively selective, as they inhibited the mammalian enzyme with greater efficacy compared to rBmAChE1.

The selectivity (Table 2) of the experimental carbarbates comparing hAChE to PpAChE was found to range from 23-fold (1) to 611-fold (7). Propoxur was found to be most selective standard carbamate with an SR of 21-fold, whereas 6 was found to have an SR of 366-fold, a 17-fold increase over propoxur. Edrophonium was found to be negatively selective for PpAChE, whereas eserine was 7.6-fold selective, 3-fold less than the least selective experimental carbamate. PS inhibitors were found to be poorly selective, with SR values near unity (Table 2). A ten-fold difference in SR was observed between the bivalent inhibitors, with donepezil being negatively selective for PpAChE and BW284c51 being non-selective (SR = 1.1).

3.4. Homology modeling and site directed mutagenesis (W384F) of rBmAChE1

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

Inhibition potencies of AChE inhibitors to the rBmAChE1 (W384F) mutant enzyme and comparison to rBmAChE1 are shown in Table 4. When compared to rBmAChE1 wildtype, the mutated rBmAChE1 (W384F) enzyme displayed a statistically significant increase in sensitivity to tacrine, BW284c51, and donepezil. Tacrine and BW284c51 were found to be 64.4-fold and 8-fold more potent to the mutated enzyme (W384F) when compared to rBmAChE1 wildtype (Table 4). Donepezil had a >40-fold increase in potency to the mutated enzyme, but an exact value could not be determined, since only 3% of the rBmAChE1 wildtype enzyme was inhibited at 100 μM. Propoxur was the only carbamate AChE inhibitor to show a statistically significant increase (1.4-fold) in IC

50 values between the wildtype and mutant enzymes. The other carbamates had activity ratios near unity (Table 4).

3.5. AChE from resistant tick strains

A number of resistant strains were collected and their AChEs cloned into cell lines and sequenced (Table 1). The strains were tested in preliminary screens against a variety of CS- and PS-directed compounds listed in Table 2, and little resistance was noted. More complete experiments were performed with compounds 1 and 6. Compound 1 was tested because we have studied it extensively over the last few years [28], and compound 6 because it was a potent in vitro inhibitor of rBmAChE1. IC

50 values for these strains were all in the low nanomolar range, and they generally showed little cross resistance to coumaphos oxon and experimental compounds 1 and 6, as shown in Table 5. Exceptions were compounds 1 and 6 in the Tuxpan #11 strain, where the resistance ratios were 2.3 and 5.2, respectively, likely due to influence of the D188E and/or E196G substitutions in the Tuxpan #11 construct (Table 1).

3.6. Mammalian toxicity studies

Estimates of LD

50 values were collected for experimental carbarbates 1, 6, and 7. LD

50 estimates for compounds 1 and 6 were 154 (n = 8) and 1732 (n = 7) mg/kg, respectively. Due to low numbers of animals used, no confidence limits for these values could be accurately calculated. For compound 7, no lethality was observed up to 2000 mg/kg (n = 2).

4. Discussion

CS-directed compounds were potent inhibitors of both enzymes, but structure–activity relationships of carbarbates differ
for tick and sandfly AChE. For rBmAChE1, the loss of activity observed for carbamates 2 and 3 when compared to 1 suggest the enzyme has difficulty accommodating the larger trialkysilyl groups of 2 and 3, and that meta side chains must be smaller in size to effectively inhibit the enzyme. In contrast, PpAChE apparently can accommodate these groups with little loss of activity (Table 2). Potency against rBmAChE1 for ortho-substituted phenyl methylcarbamates (4-6) was high, but they were less active than meta-substituted compounds when tested on PpAChE. The high potency of 6 to both rBmAChE1 and PpAChE is exceptional, as it is 7-fold more potent when compared to AgAChE (IC<sub>50</sub> 104 nM [37]), the enzyme species it was designed to target. Compound 6 was shown to be equipotent to coumaphos oxon and possess about 3-fold and 10-fold selectivity over bovine AChE and hAChE, respectively (Table 2). Selectivity was reduced to <10-fold with meta-substituted side chains, suggesting the design of future N-methylcarbamates for Bm AChE control should focus on ortho-substitutions. The excellent potency and selectivity of compound 6 for PpAChE suggests it might be a viable control method for Pp populations in locations of close proximity to humans, such as insecticide-treated nets or indoor residual spraying.

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In an effort to validate the homology models, site directed mutagenesis was performed to study the effect the W384/Y337 substitution has on the inhibitor potency to the BmAChE1 enzyme. The mutation performed was W384F, since phenylalanine possesses a smaller structural size when compared to tryptophan and a closer resemblance to tyrosine, which is found in the majority of other enzymes, including hAChE. Statistically significant increases in potency were observed with tacrine and the two bivalent inhibitors, BW284c51 and donepezil. Although the increase in potency to the mutated enzyme with tacrine and bivalent inhibitors validates the model of W384 preventing access of larger ligands to the acyl site, it does not fully account for the decreased IC50 values to those observed in other species. One would expect an additional 172-fold increase in potency for tacrine if the mutation accounted for all of the decreased inhibitor potency. Therefore, it is plausible to suggest other orthologous substitutions are present in conjunction with W384/Y337 (Bm/human numbering), allowing for a unique BmAChE1 gorge geometry and therefore, a constricted entry to the acyl site. For instance, molecular homology models suggest the T335/W286 (Bm/human numbering) substitution in the Bm peripheral site could account for a reduced sensitivity to peripheral site and bivalent inhibitors. The T335/W286 substitution in BmAChE1 could potentially disrupt the transient binding mechanism of the PS [25] and effectively reduce the potency of acyl site inhibitors through allosteric effects at the CS.

Resistant strain enzymes utilizing a number of additional mutations (Table 1) were analyzed for sensitivity to experimental carbamates. In general, these studies observed little cross resistance to either coumaphos or carbamates (Table 5), and would not be expected to cause field use failures. An exception was Tuxpan #11, which showed about 5-fold resistance to 6 at the enzyme level, and might indicate incipient resistance to this molecule. This strain carries four mutations, two of which (D188G and E196G) are unique to Tuxpan #11. It would be appropriate in future studies to analyze further the distribution and impact of these mutations on resistance.

Mouse toxicity data for propoxur, a WHO-approved carbamate for mosquito control, has a mammalian LD50 of 24 mg/kg using methods essentially identical to those employed here [38]. Thus, it was sixfold more toxic than compound 1 (154 mg/kg) to mice. The presence of longer side chains containing a sulfur or oxygen atom in the ortho position, as seen in 6 and 7, decreased the mammalian toxicity by at least 70-fold when compared to propoxur, consistent with their reduced activity against mammalian enzymes (Table 2). These data suggest the novel carbamates would be substantially safer than the currently utilized methycarbamates in pest control applications, if they provide equal or better in vivo toxicity to pest species at similar application rates.

Acknowledgments

We thank the National Institutes of Health (AI082581), the Foundation for the National Institutes of Health (GGHH-1497) through the Grand Challenges in Global Health Initiative, the United States Department of Agriculture (Specific Cooperative Agreement 108-0128-098) and Deployed War-fighter Protection Research Program, as well as Virginia Tech (the College of Science, the Department of Chemistry, and Fralin Life Science Institute) for financial support of this work. We would also like to thank Kristie Schlechte for excellent technical assistance with the tick recombinant enzymes, and Dr. James Mutunga for his part of the mouse oral toxicity studies. The USDA is an equal opportunity provider and employer.

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