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Discovery of Species-selective and Resistance-breaking Anticholinesterase Insecticides for the Malaria Mosquito

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Abstract

Great reductions in malaria mortality have been accomplished in the last 15 years, in part due to the widespread roll-out of insecticide-treated bednets across sub-Saharan Africa. To date, these nets only employ pyrethroids, insecticides that target the voltage-gated sodium ion channel of the malaria vector, *Anopheles gambiae*. Due to the growing emergence of *An. gambiae* strains that are resistant to pyrethroids, there is an urgent need to develop new public health insecticides that engage a different target and possess low mammalian toxicity. In this review, we will describe efforts to develop highly species-specific and resistance-breaking inhibitors of *An. gambiae* acetylcholinesterase (AgAChE). These efforts have been greatly aided by advances in knowledge of the structure of the enzyme, and two major inhibitor design strategies have been explored. Since AgAChE possesses an unpaired Cys residue not present in mammalian AChE, a logical strategy to achieve selective inhibition involves design of compounds that could ligate that Cys. A second strategy involves the design of new molecules to target the catalytic serine of the enzyme. Here the challenge is not only to achieve high inhibition selectivity vs human AChE, but also to demonstrate toxicity to *An. gambiae* that carry the G119S resistance mutation of AgAChE. The advances made and challenges remaining will be presented. This review is part of the special issue “Insecticide Mode of Action: From Insect to Mammalian Toxicity.”

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NOTE IN PROOF

After this manuscript was accepted, a 3.4 Å structure of *Anopheles gambiae* AChE was published by coauthors Li, Carlier, and Totrov and deposited in the PDB (PDB ID: 5×61): Han, Q.; Wong, D.M.; Robinson, H.; Ding, H.; Lam, P.C.H.; Totrov, M.M.; Carlier, P.R.; Li, J. Crystal structure of acetylcholinesterase catalytic subunits of the malaria vector *Anopheles gambiae*. *Insect Science*, **2017**, *00*, 1–4, DOI 10.1111/1744-7917.12450.

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CONFLICT OF INTEREST

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Acetylcholinesterase; sulfhydryl reagents; cysteine; carbamates; trifluoromethyl ketones; difluoromethyl ketones

1. INTRODUCTION

1.1. Vector Control to Reduce Malaria Transmission

Malaria remains a global killer, responsible for an estimated 438,000 deaths in 2015 [1], and children under 5 years old represent 70% of the mortality. Malaria is caused by parasites of the genus *Plasmodium*, and transmission of the parasite occurs *via* blood-feeding by adult female *Anopheles* mosquitoes. The main vector of malaria in sub-Saharan Africa is *Anopheles gambiae* (*An. gambiae*). Because this species has a marked preference for nighttime biting, effective vector control can be accomplished by deploying contact insecticides within dwellings, either by applying them to the inside walls (indoor residual spraying or IRS) or to bednets (insecticide-treated nets or ITNs). At present the mosquitocides approved by the World Health Organization are directed to only two biological targets: the voltage-gated sodium channel, and acetylcholinesterase (AChE, EC 3.1.1.7) [2]. Insecticides targeting the voltage-gated sodium ion channel include DDT and a series of pyrethrin-derived synthetic compounds called pyrethroids; a number of pyrethroids are approved for use on ITNs and for IRS.

WHO-approved mosquitocides targeting AChE fall into two structural classes: organophosphates (which are typically phosphorothioates) and methylcarbamates (Fig. 1).

To date, anticholinesterase mosquitocides have only been approved for IRS.

1.2. Addressing the Challenge of Pyrethroid-resistant *Anopheles gambiae*

Since 2000, the estimated number of annual global malaria deaths has declined 48%, [1] and the widespread deployment of ITNs have made a major contribution to this reduction [3–5]. Current ITNs rely exclusively on pyrethroid insecticides, and the growing resistance of *Anopheles* to pyrethroids is great cause for concern [6]. To address this challenge it would be ideal to develop new selective insecticides that do not engage the target of existing vector control compounds, and/or circumvent known resistance mechanisms. This review article will address efforts made to design new anticholinesterase mosquitocides that have low mammalian toxicity and might allow them to be deployed on ITNs. Given the well-known mammalian toxicity of many AChE inhibitors, [7–9] this is a challenging goal. To achieve low mammalian toxicity, an ideal mosquitocidal AChE inhibitor would be a poor inhibitor of human AChE (*hAChE*). Thus, high levels (>100-fold) of target site inhibition selectivity would be desirable. A second and parallel goal is to design new anticholinesterase mosquitocides that would be effective against organophosphate- and carbamate-resistant strain *Anopheles*. These efforts were guided by important advances in the understanding of *An. gambiae* AChE (*AgAChE*).

2. AN. GAMBIAE ACETYLCHOLINESTERASE

2.1. Functional AgAChE is Encoded by the *ace-1* Gene

The determination of the X-ray crystal structure of AChE from the electric fish *Torpedo californica* (*TcAChE*) in 1991 was a watershed moment in cholinesterase biology, providing great insight into the structure and function of this serine hydrolase [10]. Since that date hundreds of X-ray crystal structures of AChE have been publicly deposited, but at present no X-ray structures of a mosquito AChE are available. Interestingly, mosquitoes have two genes encoding acetylcholinesterases: *ace-1* and *ace-2* [11, 12]. The common house mosquito (*Culex pipiens*) expresses both enzymes [13,14]. The fruit fly (*Drosophila melanogaster*) expresses a single AChE encoded by *ace-2*, and in 2000 Sussman and co-workers successfully determined the X-ray crystal structure of *D. melanogaster* AChE (*DmAChE*, PDB ID 1q09, 2.7 Å) [15]. In 2002, Weill and co-workers determined that *An. gambiae* expresses *ace-1* AChE rather than the *ace-2* AChE observed in *D. melanogaster*. The *ace-2* AgAChE has a 31 amino acid insertion relative to *ace-1* AgAChE, [12] and comparison of the sequences of *DmAChE* (*ace-2*) and AgAChE (*ace-1*) revealed only 52 % sequence similarity [12]. Thus, *DmAChE* (to date, the only insect AChE X-ray structure in the PDB) is not a good model for AgAChE. Using genetic techniques, Weill subsequently determined that organophosphate and carbamate resistance in *An. gambiae* is associated with a single amino acid mutation: serine for glycine at position 119 (*Torpedo californica* numbering) [16]. Glycine119 of the catalytic domain is one of the key residues in the so-called “oxyanion hole” of AChE, providing an N–H hydrogen bond to the developing negative charge on the carbonyl oxygen of acetylcholine as it binds to the catalytic serine. The increased steric size of the serine side chain (CH₂OH) relative to that of glycine (H) was proposed to be responsible for carbamate and organophosphate insensitivity in G119S AgAChE [17]. A simple PCR test was later developed to determine the resistance status of single mosquitoes (RR, RS, SS), which demonstrated that sensitivity of AChE in mosquito homogenate was well-correlated to resistance status [17]. The G119S mutation has also been detected in *Culex* mosquitoes [16], and subsequent studies revealed additional resistance mutations (F331W [18] and F290V [19]) within this genus. However, to date, G119S is the sole mutation associated with organophosphate and carbamate resistance in *An. gambiae*.

2.2. Expression, Purification, and Characterization of AgAChE (WT and G119S)

The first reported recombinant expression of AgAChE was realized in S2 cell lysate, using vectors encoding the catalytic subunit of wild-type (WT)[16,20] and G119S[16]. AgAChE. Studies with the recombinant WT and G119S AgAChE demonstrated propoxur-insensitivity of the G119S enzyme, [16] and confirmed the poor enzymatic selectivity of propoxur for AgAChE over human AChE (*hAChE*) [20]. Two AChE inhibitors (tacrine and galantamine), approved for treatment of Alzheimer’s disease, were shown to have poor AgAChE/*hAChE* inhibition selectivity, and a potent peripheral site inhibitor of *hAChE* (0.4 nM IC₅₀, Fasciculin II, 4 kDa peptide) was shown to cause no measurable inhibition of AgAChE at 100 nM [20]. Purified WT AgAChE (catalytic domain) suitable for kinetic characterization was first obtained by expression of the catalytic subunit in Sf21 cells [21] *K_m*, specific activity at saturating substrate (*V_{max}*, U/mg), and *k_{cat}* were determined for the standard substrate analog acetylthiocholine (ATCh) and three other analogs. Kinetic inhibition

constants were also determined for carbaryl, and the active metabolites (oxons) of malathion and parathion (Table 1) [21].

This study also documented substrate inhibition of *AgAChE* at high concentrations of ATCh, a common feature of many AChE species. A later study used yeast cells to express the catalytic domains of both WT and G119S *AgAChE* [22]. This study confirmed the high $V_{\max}$ of WT *AgAChE* (2,700 U/mg, cf. 5,400 U/mg for *hAChE*), and demonstrated that the G119S mutation has a dramatic effect on substrate processing (Table 2). The G119S mutation has only a small effect on the K_m value of ATCh (cf. 53.8 μ M for WT and 128 μ M for G119S), but dramatically reduces k_{cat} for the G119S mutant (1.8×10^5 and 5.3×10^3 min^{-1} , respectively).

Consequently, the G119S enzyme has only 1.2% of the catalytic efficiency (k_{cat}/K_m) of the WT enzyme. Thus, it is to be expected that some substrate analog inhibitors of AChE (organophosphates, carbamates) may also show reduced sensitivity to G119S *AgAChE*. Recently, *AgAChE* (WT and G119S) and *Aedes aegypti* AChE (WT, *AeAChE*) were also expressed in Sf9 cells [23]. Similar kinetic parameters (K_m , k_{cat}) were found for *AgAChE* and *AeAChE*. As reported in the earlier study, the G119S mutation in *AgAChE* caused near doubling in K_m , and insensitivity to inhibition by propoxur. However, the measured k_{cat} (7.4×10^3 min^{-1}) of the WT *AgAChE* enzyme was significantly lower than that obtained by other groups (cf. Table 2) [21,22]. Finally, transcript levels of *ace-1* and *ace-2* *AgAChE* were studied in *An. gambiae* in different developmental stages and in three body parts [24]. *Ace-1* *AgAChE* mRNA levels were always significantly higher than those of *ace-2* *AgAChE*. These and other observations are consistent with the long-standing hypothesis [16,17] that only *ace-1* *AgAChE* plays a major role in hydrolyzing synaptic ACh in *An. gambiae*.

3. DEVELOPMENT OF COVALENT INHIBITORS OF AN. GAMBIAE AChE THAT TARGET C286

3.1. Background

In their 2006 review of acetylcholinesterase structure and function, Radi and Taylor noted that several insect AChEs possess additional Cys residues not found in mammalian AChE, and that these most likely remain unpaired; alignment of 125 cholinesterases reveals that C286 is one such residue in *An. gambiae* (note *Torpedo californica* AChE numbering is C288) [11]. Near the same time, Pezzementi published a sequence alignment that highlighted the presence of homologous free cysteines in the acyl pockets of several invertebrate AChEs: *ace-1* *AgAChE*, *Culex pipiens* *ace-1* AChE, three aphid AChEs, and *Branchiostoma floridae* (an eel-like cephalochordate) AChE [25]. Inactivation of several mutants of *B. floridae* AChE by the sulfhydryl reagents 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide (NEM) was observed, suggesting that covalent modification of a free Cys was responsible for the loss of enzymatic activity. These studies then provided an explanation for the early observation that DTNB rapidly inactivates aphid AChE [26]. In 2006 Pang independently published AChE sequence alignments that indicated the presence of a free Cys in *An. gambiae* AChE (C286), as well as in 16 other invertebrate AChEs [27]. Multiple molecular dynamics simulations were used to generate a computationally refined

homology model of *AgAChE* (PDB ID: 2AZG) that suggested that C286 was indeed surface accessible and that covalent modification of it could impede substrate processing [27]. He was the first to propose C286-targeting agents as insecticides for *An. gambiae* [27]. Similar calculations were performed for the AChEs of two crop pests (the greenbug and English grain aphid), which suggested that the homologous Cys289 in these species could serve as an insecticidal target [28].

3.2. Experimental Confirmation of the Hypothesis

The first published attempt to inhibit *AgAChE* by covalent modification of C286 involved iodoacetamide-tethered tacrines **1a–h** (Fig. 2) [20]. The design of compounds **1a–h** was inspired by Pang's 2006 report, [27] and by methanethiosulfinate-tethered tacrine **2**, that successfully inhibited the H287C mutant of *hAChE* [29]. Compound **2** was designed to allow docking of the tacrine unit in the active site of AChE, [30] and the tethered methanethiosulfinate moiety would then be well-positioned to react with the engineered C287. Among sulfhydryl-reactive functional groups, methanethiosulfinates are among the most reactive, [31] and incubation of **2** (6 mM, 30 min) with H287C *hAChE* caused a >99.5% loss in enzyme activity [29]. Iodoacetamides are less-reactive sulfhydryl reagents, and tacrine-tethered iodoacetamide **1a–h** proved to be potent inhibitors of both *hAChE* and *AgAChE* (10 min IC₅₀ = 0.1 – 340 nM). Unexpectedly, most of the compounds were 20- to 100-fold selective for inhibition of *hAChE* over *AgAChE*, and over a 10 min period at 10 μ M, the tacrine iodoacetamides **1a–h** did not evidence progressive inhibition of *AgAChE*. Thus, it was concluded that **1a–h** were reversible non-covalent inhibitors of both *AgAChE* and *hAChE*, and that covalent modification of C286 in *AgAChE* had not been realized with these compounds.

The Pang group subsequently reported trimethylammonium-tethered methanethiosulfinates **3a–n** designed to target insect AChEs (Fig. 3). Quaternary groups are well known to impart affinity for the AChE active site, [30] and the trimethylammonium group maximized resemblance to acetylcholine. Incubation of greenbug aphid (*Schizaphis graminum*) homogenate with **3l** ($n = 18$, 6.0 μ M, 1 h) resulted in 99% inhibition of AChE activity; under the same conditions, no inhibition of *hAChE* was seen [32]. Partial rescue of AChE activity in the **3l**-inhibited aphid enzyme was achieved by subsequent treatment with 2-mercaptoethanol (100 mM, 2–6 h). These observations suggested that the inhibition of aphid AChE was due to covalent modification of the exposed free Cys residue, which is absent in *hAChE*. Interestingly, the shorter tether analog **3g** ($n = 13$) caused irreversible inhibition of *hAChE*, which was attributed to possible covalent modification of the catalytic serine [32].

The trimethylammonium-tethered methanethiosulfinates **3a–n** were then assessed against the AChE present in the homogenate of three mosquito species: *An. gambiae*, *Aedes aegypti* (vector for yellow fever, Dengue, Chikungunya, Zika), and *Culex pipiens* (vector for St. Louis and Japanese encephalitis, West Nile virus). Incubation with 6 μ M **3d–n** for 1 h caused >95% inhibition of *AgAChE*, 75–90% inhibition of *C. pipiens* AChE, and 60–80% inhibition of *Ae. aegypti* AChE (Fig. 3) [33]. Treatment of **3l** ($n = 18$)-treated *AgAChE* with 2-mercaptoethanol again led to substantial recovery of enzymatic activity. The failure of this reagent to achieve higher recovery of enzyme activity (through deconjugation of the

covalently bound inhibitor from C286) was attributed to the observed high sensitivity of apo-*AgAChE* to this reagent. Progressive inhibition of the AChEs present in beetle and cockroach homogenate was also demonstrated with **3l**, by following the decrease in enzyme velocity with increasing incubation time at 6 μM over 70 min [34]. To assess the level of reactivity needed to ligate Cys286, the less reactive 1° bromide **4** was then assessed as an inhibitor. But following a 2.5 h incubation at 1 μM , only a 10% reduction in *AgAChE* activity was observed [33].

Second-generation Cys-targeting inhibitors of *AgAChE* were disclosed in 2013 [35]. These compounds featured *N*-alkylpyridinium or basic *N*-alkylpiperidine groups to confer affinity for the AChE active site, tethered to maleimide (**5**, **7**) or succinimide **6**, **8**) groups. Progressive inhibition of recombinant *AgAChE* over 15 min was seen with 6 nM **5b**, and 200 nM **7a**, consistent with Michael addition of Cys286 to the maleimide units. Apparent second-order inactivation rate constants (k_{inact}/K_i) were determined for **5b**, **7a**, and paraoxon. In particular, compound **5b** inactivates *AgAChE* extremely fast, with $k_{\text{inact}}/K_i = 275,000 \text{ mM}^{-1} \text{ min}^{-1}$, a remarkable 239-fold faster than paraoxon. Finally, that **5b** covalently modified Cys286 in *AgAChE* was convincingly demonstrated by Nano-LC-ESI-MS/MS analysis of recombinant *AgAChE* treated with **5b** [35]. Succinimide analogs **6b** and **8a** lack the Michael acceptor functionality needed to covalently bind to C286, and no progressive inhibition was seen with 300 nM **6b** or 10 μM **8a**. Furthermore, as expected, no progressive inhibition of *hAChE* was observed over 15 min upon exposure to 200 nM **5b** or 20 μM **7a**.

3.3. Challenges Remaining for Cys-targeting Insecticides

All of the observations described above are consistent with covalent modification of C286 as the mechanism of inhibition of *AgAChE* by compounds **5b** and **7a**. It has therefore been demonstrated that rapid and selective inhibition of *AgAChE* over *hAChE* can be achieved with the appropriate sulfhydryl reagent. However, to date no studies of the insecticidal activity of Cys-targeting AChE inhibitors have been published [36]. Thus, despite the extremely promising *in vitro* results achieved, the practicality of this approach for vector control remains to be demonstrated. As will be emphasized below, insecticidal efficacy is not determined solely by the potency of AChE inhibition, but also by absorption, distribution, metabolism, and excretion.

4. DEVELOPMENT OF SPECIES-SELECTIVE AND RESISTANCE-BREAKING INHIBITORS OF AN. GAMBIAE AChE THAT TARGET THE CATALYTIC SERINE (S199)

4.1. Background

Inhibitors that target the catalytic serine of AChE (S200 in *Torpedo californica* AChE (*TcAChE*), S199 in *AgAChE*) have been in widespread use as insecticides since the 1940s [7,8]. As mentioned in Section 1.1, these compounds fall into two main classes, organophosphates and carbamates, and early on both were used to control vector mosquitoes, including *Anophelines* [37,38]. Perhaps due to concerns regarding the

relationship of organophosphate insecticides with a delayed neuropathy, [39] to our knowledge there has been no published work directed toward development of species-selective organophosphates for *An. gambiae*. Therefore, the section below will address the development of new carbamates and fluorinated methylketones to selectively inhibit *AgAChE* over *hAChE*, and to potentially inhibit the G119S carbamate-insensitive *AgAChE*.

Both methylcarbamates and trifluoromethylketones are substrate analog inhibitors; like acetylcholine (ACh) they form a covalent adducts with the catalytic serine, which for *AgAChE* is S199 (Fig. 4).

In the case of methylcarbamates, the methylcarbamoylated enzyme hydrolyzes much more slowly than the acetyl enzyme formed from normal processing of ACh (Fig. 4A). For trifluoromethylketones a stable hemiketal is formed, which resembles the first tetrahedral intermediate formed by ACh and *AgAChE* en route to the acetyl enzyme (Fig. 4B). For this reason, trifluoromethylketones are sometimes considered transition state analogs. X-ray crystal structures of *T. californica* and mouse AChE have demonstrated these adducts for both carbamates[40–42] and trifluoromethylketones[43–45].

Given that the catalytic triad is conserved across all species of AChE, it might seem unlikely that a molecule that targets the catalytic serine could achieve high selectivity for inhibition of a particular species of AChE. However, studies by Silman and co-workers demonstrated that the Alzheimer therapeutic drug rivastigmine inactivates *hAChE* 1600-fold faster than it inactivates *TcAChE* (Fig. 5) [41]. Note that rivastigmine features the carbamate warhead to covalently modify the catalytic serine. Thus, highly selective inhibition of *AgAChE* by a catalytic serine-targeting agent should be possible, especially since *AgAChE* shares only 49% sequence identity with *hAChE* [20].

4.2. Species-selective, Insecticidal Methylcarbamates for *An. gambiae*

To search for compounds that might selectively inhibit *AgAChE*, Carrier and co-workers prepared a series of 34 methylcarbamates derived from 2- and 3-substituted phenols [46]. Compounds of particular interest are depicted in Fig. 5; mosquitocidal carbamates propoxur and bendiocarb serve as references and are depicted in Fig. 1. The apparent second-order rate constants (k_i) for inactivation of recombinant *AgAChE* and *hAChE* by these compounds were then measured (Table 3).

As can be seen, propoxur and bendiocarb offer low selectivity for inhibition of *AgAChE* vs *hAChE* ($S = 16$ - and 7.6 -fold, respectively). Methylcarbamates of 3-substituted phenols also inhibit *AgAChE* with only ~ 10 -fold selectivity, correcting an earlier report that indicated high inhibition selectivity of **10** [20]. However, methylcarbamates with select γ -branched 2-substituents can offer excellent selectivity for inhibition of *AgAChE* over *hAChE*. For example, compounds **11** and **13** ($A = CH_2$ and S , respectively) both offer greater than 100-fold selectivity, and analog **12** ($A = O$) offers greater than 500-fold selectivity for inhibition of *AgAChE* relative to *hAChE* [46–48].

The toxicity of these compounds to fully susceptible G3 strain adult female *An. gambiae* was then determined in two assays. The tarsal contact assay involves 1 h exposure of adult

(3–5 day old) mosquitos to filter papers pre-treated with ethanolic solutions of the candidate insecticide [49]. Toxicity in this assay is measured by the concentration of the solution that causes 50% mortality after 24 h (LC_{50} , $\mu\text{g/mL}$). The topical application assay involves placement of 200 nL of an insecticide solution on the dorsal thorax of cold-anesthetized adult female *An. gambiae* [50]. Insects are revived by warming to room temperature, and toxicity in this assay is measured by the dose that causes 50% mortality after 24 h (LD_{50} , ng/insect). As can be seen in Table 3, the non-selective methylcarbamate **9** approximates the toxicity of propoxur in the tarsal contact assay; however highly species-selective inhibitors **11–13** are significantly less toxic. In the topical assay, the LD_{50} values of **12** and **13** were determined to be 25- and 3-fold higher than that of propoxur. Therefore, although their toxicities are less than that of propoxur and bendiocarb, species-selective inhibitors **12** and **13**, do show significant mosquitocidal activity against adult *An. gambiae*. Subsequent studies also showed significant insecticidal activity of these compounds against adult *Aedes aegypti* and *Culex quinquefasciatus* (the vector of lymphatic filariasis) [51].

These data also reconfirm that insecticidal activity is not a simple function of $AgAChE$ inhibition potency. Although the structures of propoxur and **12** are closely related, and their $AgAChE$ k_i values are identical within experimental error, propoxur is significantly more toxic than **12**. In addition to differences in metabolic detoxification, [52] the physicochemical characteristics of each $AChE$ inhibitor will impact its transport to the mosquito CNS. To account for these factors, a predictive model of $\log(LC_{50})$ was developed for 24 aryl methylcarbamates that took into account not only $\log(AgAChE$ $k_i)$, but also molecular volume and polar surface area [47]. As expected, $\log(LC_{50})$ decreased with increasing $\log(AgAChE$ $k_i)$, and decreased with decreasing molecular volume (which is a surrogate of molecular weight). Unexpectedly, $\log(LC_{50})$ also decreased with increasing polar surface area.

The potency of these species-selective carbamates to inhibit the $AChE$ of several non-target organisms was studied [53]. High (200- to 600-fold) selectivity for **12** and **13** was seen for mouse and chicken $AChE$, but lower (2- to 33-fold) selectivity was seen against the $AChE$ of aquatic organisms (electric eel, *Torpedo californica*, water flea). Furthermore, compounds **12** and **13** were similar to bendiocarb in their toxicity to the water flea (*Daphnia magna*). In contrast, **12** and **13** were less toxic than bendiocarb to a crop pest (European corn borer, *Ostrinia nubilalis*), [51] and their mouse oral LD_{50} values were measured to be >2000 and 1700 mg/kg, respectively [54]. These values are >13- and 11-fold higher than that of propoxur, indicating that **12** and **13** might possess enhanced mammalian safety as a consequence of their high inhibition selectivity.

4.3. Resistance-breaking Carbamate and Carboxamide Mosquitocides for *An. gambiae* that Potently Inhibit G119S $AgAChE$

As mentioned above, the G119S mutation confers insensitivity towards carbamate and organophosphate inhibitors, and consequently *Anopheles* carrying G119S $AChE$ are resistant to these inhibitors. Enzymatic sensitivity ratios (SR) and toxicological resistance ratios (RR) for several $AChE$ inhibitors are shown in Table 4, where propoxur, bendiocarb, and the species-selective carbamates **12** and **13** do not measurably inactivate G119S

AgAChE, giving SR values of 3,800 to 40,000 [47]. Tarsal contact toxicity was then determined for these compounds against adult female (3–5 d old) Akron strain *An. gambiae*. This strain of mosquitoes carries the G119S mutation of AgAChE, and LC₅₀ values for propoxur and bendiocarb are greater than 5,000 µg/mL, leading to RR values > 130 (Table 4).

In contrast, the agricultural insecticide aldicarb measurably inhibited G119S AgAChE ($k_i = 3.15 \pm 0.08 \text{ mM}^{-1} \text{ min}^{-1}$) and had an SR of only 4.2 [22]. Aldicarb proved quite toxic to Akron strain *An. gambiae* (LC₅₀ = 32 µg/mL) and had an RR value of 0.5, meaning it was more toxic to Akron than the G3 strain. As shown in Fig. 6, aldicarb is an oxime methylcarbamate, and consequently possesses a leaving group that is slimmer than the phenols present in propoxur, bendiocarb, **12**, and **13**.

Molecular modeling studies of the tetrahedral intermediates of aldicarb with WT and G119S AgAChE showed that aldicarb suffered no steric interactions in binding to the catalytic serine (S199) of G119S AgAChE [22]. In contrast, 3-butylphenol-derived methylcarbamate **9** was projected to suffer unfavorable steric interactions with S119 on binding to S199 of G119S AgAChE. This observation prompted the exploration of a series of methylcarbamates wherein the phenol unit was replaced with *N*-alkylpyrazol-4-yl alcohol (Fig. 6). As can be seen in Table 4, pyrazol-4-yl methylcarbamates **14** and **15** very rapidly inactivated WT AgAChE, and they also rapidly inactivated G119S AgAChE, with k_i values ranging from 137 to 290 mM⁻¹ min⁻¹ [22]. Consequently the SR values of these compounds (30–32) was much more favorable than that of the phenol carbamates. Consistent with their rapid inhibition of G119S AgAChE, these compounds proved quite toxic to Akron strain *An. gambiae*, with RR = 0.8 to 1.5 [22]. The considerably lower RR value for these compounds relative to their SR values again points to the role of metabolism and transport in modulating toxicity. In addition, these compounds potently inhibited hAChE, suggesting that further optimization would be required to achieve a resistance-breaking, species-selective AgAChE inhibitor.

Methylcarbamates derived from 5-alkyl-isoxazol-3-yl alcohols were also explored, but proved to be unstable. Preparation of the corresponding dimethylcarbamates (e.g. **16–17**) led to the concurrent formation of dimethylcarboxamides (e.g. **18–19**). Both compound classes offered potent inhibition of the G119S enzyme, and compounds **16–19** had G119S AgAChE k_i values ranging from 10.6 to 30.6 mM⁻¹ min⁻¹ [55]. Not surprisingly, these compounds were toxic to Akron *An. gambiae*, with LC₅₀ values ranging from 58 to 296 µg/mL. As was the case with the pyrazol-4-yl methylcarbamates **14–15**, potent inhibition of hAChE led to very poor inhibition selectivity. The toxicological resistance ratios (RR) were also much lower (0.5–2.0) than would have been expected based on enzyme sensitivity ratios (14–360) [55].

Oxime carbamates derived from trifluoromethylketones have long been known to be insecticidal, [56] and thus a series of these compounds was investigated for their resistance-breaking activity and species-selectivity [57]. In particular, trifluoromethylketone oxime methylcarbamates **20–21** were found to rapidly inactivate both WT and G119S AgAChE, with SR values of 50–53. These compounds were quite toxic to Akron strain *An. gambiae*,

and had RR values of 1.8 – 4.0. Unfortunately, these compounds also rapidly inactivated *hAChE*. Accordingly, this compound class did not appear promising.

4.4. Difluoromethylketone Inhibitors that Potently Inhibit G119S *AgAChE*

Trifluoromethyl ketones are well-known as inhibitors of *AChE*[58,59] and juvenile hormone esterase [60]. Despite the remarkable (i.e. picomolar) potencies that can be achieved [61], with few exceptions [62] this class of compounds has received little attention as insecticides. Difluoromethyl and fluoromethyl ketones are relatively unexplored as anticholinesterases, [63] but have been used as serine protease inhibitors[64–66]. Carlier and co-workers investigated a series of fluorinated methylketones based on the 3-*t*-butylphenyl and *N*-alkylpyrazol-4-yl scaffolds present in methylcarbamates **9** and **14–15** [67]. As these inhibitors are reversible covalent inhibitors of *AChE* (Fig. 4B), and since trifluoromethylketones can be slow, tight-binding inhibitors, [61] inhibition potency was determined by measuring enzyme activity following 10 min and 60 min incubations. IC₅₀ values following 60 min incubations are shown in Table 5. Except for compound **22** at *AgAChE* and *hAChE*, and **26** at G119S *AgAChE*, all compounds achieved steady state inhibition within 10 min. Surprisingly, difluoromethyl ketones were similar in potency for inhibition of *AgAChE* as the corresponding trifluoromethyl ketones (cf. **23** & **22**, and **26** & **25**).

Fluoromethyl ketones **24** and **27** were much less potent than the di- and trifluoromethyl ketones, but fluoromethyl ketone **27** did achieve a sub-micromolar IC₅₀ value at *AgAChE*. Potencies of **23–27** for inhibition of the G119S mutant *AgAChE* were significantly reduced relative to their potencies for the WT enzyme, as evidenced by the SR values of 9 to 2500. Yet interestingly, difluoromethyl ketone **26**, after a 23 h incubation, gave a 25 nM IC₅₀ value for G119S *AgAChE*[67]. Selectivity of these compounds for inhibition of *AgAChE* vs *hAChE* ranged from inverse (*S* = 0.3, **22**) to moderate (*S* = 29, **26**). Unfortunately, none of these compounds were appreciably toxic to G3 strain adult *An. gambiae* in the tarsal contact assay: the LC₅₀ value of **25** was ~ 1,000 µg/mL. Thus, compound **25** was 25-fold less toxic than propoxur, despite the fact that its 60 min IC₅₀ value was 64-fold lower. Difluoromethyl ketone **26** similarly caused no mosquito mortality at 1,000 µg/mL. Thus, this study provided another example that potent target engagement is only one of several factors needed to achieve good insecticidal efficacy.

4.5. Challenges Remaining for Catalytic-Serine-Targeting Mosquitocides

The discovery of methylcarbamate inhibitors **11–13** demonstrates that it is possible to design catalytic serine inhibitors that have high (110- to 530-fold) potency to inhibit *AgAChE* over *hAChE*. Compound **13** (130-fold selective) has topical toxicity against susceptible adult *An. gambiae* within 3-fold of that of propoxur, a compound approved by the WHO for IRS. However, phenol-derived methylcarbamates like **13** are poor inhibitors of the G119S resistant mutant *AgAChE*, and consequently have low toxicity toward Akron strain *An. gambiae* that carries this mutation. A series of carbamates and carboxamides containing smaller leaving groups were explored (**14–21**) to achieve potent inhibition of the G119S *AgAChE*, and excellent toxicity to Akron was achieved. However, these compounds offered poor selectivity for inhibition of *AgAChE* vs *hAChE*. A series of fluorinated methyl ketones

were then investigated, and although difluoromethyl ketone **26** potently inhibited both *AgAChE* and G119S *AgAChE*, it was not appreciably toxic to *An. gambiae*. Thus, to date it has not been possible to combine in one molecule the desired properties of i) resistance-breaking insecticidal action and ii) high enzymatic inhibition selectivity.

5. SPECIFIC INHIBITORS OF G119S *AN. GAMBIAE* AChE

To search for compounds that might preferentially inhibit carbamate-insensitive G119S mutants of *Culex pipiens* AChE (*CpAChE*) and *AgAChE*, Weill and coworkers screened a 3,000-compound library against both WT and G119S *CpAChE*. Downsampling and purchase of additional analogs led to the identification of seven barbaturic acid-furan or –thiophene analogs that inhibited G119S *CpAChE* 10-fold more strongly than WT *CpAChE* (e.g. **28**) [68].

Compound **28** inhibited G119S *CpAChE* 7-fold more potently than WT *CpAChE*. Organophosphate-susceptible and organophosphate resistant strain *C. pipiens* and *An. gambiae* larvae were then treated with compound **28** and analogs. Whereas **28** had similar toxicity to organophosphate-resistant and susceptible *C. pipiens* larvae, it was 7-fold more toxic to organophosphate-resistant *An. gambiae* (Acerkis strain) than to susceptible *An. gambiae* (Kisumu strain) [68].

CONCLUSION

Though highly selective inhibitors for *AgAChE* that minimally engage *hAChE* have been identified (e.g. **5b**, **7a**, **11-13**), and although carbamates and carboxamides that are highly toxic to carbamate-resistant Akron strain *An. gambiae* have been discovered (e.g. **14-21**), compounds that possess both qualities remain elusive. Furthermore, the generally weak correlation of *AgAChE* inhibition potency to *in vivo* toxicity against adult *An. gambiae* seen in studies of serine-targeting AChE inhibitors highlights the need to better understand the structural determinants of insecticide absorption, distribution, metabolism, and excretion. At the present moment, the ideal anticholinesterase insecticide that could serve as an alternative active ingredient for ITNs is not in hand.

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Fig. (1).
Anticholinesterase mosquitoicides approved for IRS by the World Health Organization
Pesticide Evaluation Scheme (WHOPES, July 2016)

Fig. (2). Iodoacetamide-tethered tacrines **1a–h** as potential C286-targeting mosquitocides for *An. gambiae* [20].

Fig. (3).

Cys286-targeting inhibitors of insect AChE (and Cys-unreactive controls) developed by the Pang Laboratory.

Fig. (4).
Highly simplified schematics of A) methylcarbamate and B) trifluoromethyl ketone inhibition of *AgAChE*, and their relation to normal processing of ACh

Fig. (5).

The Alzheimer's disease therapeutic drug rivastigmine and selected methylcarbamates **9-13** studied for selective inhibition of *A*_gAChE by Carlier and co-workers [47].

Fig. (6).

Aldicarb, small-core carbamates (**14-17**), dimethylcarboxamides (**18-19**), and trifluoromethylketone oxime carbamates (**20-21**) with activity against G119S *AgAChE* and toxicity to Akron strain *An. gambiae*.

Fig. (7).
A selective inhibitor of G119S *CpAChE* vs WT *CpAChE* and selective larvicide for organophosphate-resistant *An. gambiae*.

Table 1

Kinetic model for covalent inhibition of *AgAChE* and selected measured inhibition constants [21].

$E + I-X \xrightleftharpoons{K_d} E \cdot I-X \xrightarrow{k_2} E-I + X$ $\xrightarrow{k_i}$			
Inhibitor	K_d^a (μM)	k_2 (min^{-1})	k_i^b ($\text{mM}^{-1} \text{min}^{-1}$)
malaoxon	5.85 ± 0.79	1.37 ± 0.14	242 ± 8
paraoxon	1.00 ± 0.14	0.79 ± 0.07	830 ± 5
carbaryl	4.7 ± 1.6	1.84 ± 0.37	440 ± 2

^aNote that K_d was determined from inhibition kinetics and not from equilibrium binding of inhibitors to *AgAChE*. Thus, the measured parameter is a composite Michaelis-Menten type constant that bears some dependence on the magnitude of k_2 .

^bApparent second-order rate constant for enzyme inactivation.

Table 2

Comparison of kinetic constants of recombinant WT *AgAChE*, G119S *AgAChE* and *hAChE*, and comparison of K_m values from G3 (susceptible) and Akron (resistant) *An. gambiae* homogenate [22].

Enzyme	K_m	V_{max}	k_{cat}	k_{cat}/K_m
	(μM)	(U/mg) ^a	(min ⁻¹) ^b	(min ⁻¹ mM ⁻¹)
<i>AgAChE</i> -WT	53.8 ± 1.4	2,700 ± 100	1.8 ± 0.1 * 10 ⁵	3.3 ± 0.2 * 10 ⁶
G3 ^c homogenate	47.3 ± 2.1			
<i>AgAChE</i> -G119S	128 ± 3	83 ± 11	5.3 ± 0.7 * 10 ³	0.042 ± 0.007 * 10 ⁶
Akron ^d homogenate	109 ± 8			
<i>hAChE</i> ^e	201 ± 11	5,600 ± 100	3.6 ± 0.1 * 10 ⁵	1.8 ± 0.1 * 10 ⁶

^a Enzyme specific activity at saturating substrate concentrations; 1 unit (U) = 1 μ mol ATCh processed per minute.

^b Turnover numbers (k_{cat}) were determined based on V_{max} and the calculated molecular mass of the enzyme catalytic subunit (64.1 kDa for *AgAChE*, 64.7 kDa for *hAChE*).

^c G3 strain *An. gambiae* carries WT *AgAChE* and is carbamate-susceptible.

^d Akron strain *An. gambiae* carries G119S *AgAChE* and is carbamate-resistant.

^e Recombinant *hAChE*, Sigma C1682.

Table 3

AChE inactivation rate constants, inhibition selectivity, and mosquito toxicity of select carbamate inhibitors [46,47].

Compound	$AgAChE\ k_i\ mM^{-1}\ min^{-1}$	$hAChE\ k_i\ mM^{-1}\ min^{-1}$	S^a	G3 Strain Tarsal contact $LC_{50}\ \mu g/mL\ (95\%CI)$	G3 strain Topical $LD_{50}\ ng/insect\ (95\%CI)$
propoxur	266 ± 9	17.0 ± 0.4	16 ± 1	39 (32–45)	3.2 (2.4–4.2)
bendiocarb	839 ± 22	111 ± 5	7.6 ± 0.4	16 (14–17)	0.74 (0.52–0.97)
9	1510 ± 110	126 ± 3	12 ± 1	37 (14–60)	4.5 (3.6–5.4)
10	648 ± 29	67.8 ± 2.6	9.6 ± 0.6	169 (162–176)	nd ^b
11	75.3 ± 2.7	0.75 ± 0.03	100 ± 5	>1000	nd ^b
12	255 ± 12	0.48 ± 0.12	530 ± 130	>1000	81 (64–94)
13	1850 ± 100	14.5 ± 1.5	130 ± 15	>1000	10 (8–12)

^aInhibition selectivity (S) = $[k_i(hAChE)]/[k_i(AgChE)]$.

^b,"nd" signifies "not determined."

Table 4

Inactivation rate constants, enzyme sensitivity ratios (SR), mosquito toxicity, and resistance ratios (RR) for selected methyl carbamates, dimethylcarbamates, and dimethylcarboxamides.

Compound	AgAChE k_i mM ⁻¹ min ⁻¹	G119S AgAChE k_i mM ⁻¹ min ⁻¹	Enzyme sensitivity ratio (SR) ^a	G3 Strain Tarsal contact LC ₅₀ µg/mL (95%CI)	Akron Strain Tarsal contact LC ₅₀ µg/mL (95%CI)	Toxicological resistance ratio (RR) ^b	hAChE k_i mM ⁻¹ min ⁻¹
propoxur	266 ± 9	<0.037 ± 0.007	7200 ± 1400	39 (32–45)	>5000	>130	17.0 ± 0.4
bendiocarb	839 ± 22	<0.055 ± 0.007	15000 ± 2000	16 (14–17)	>5000	>130	111 ± 5
12	255 ± 12	<0.066 ± 0.015	3800 ± 900	>1000	nd	nd	0.75 ± 0.03
13	1850 ± 100	<0.046 ± 0.019	40000 ± 17000	>1000	nd	nd	0.48 ± 0.12
aldicarb	13.3 ± 0.3	3.15 ± 0.08	4.2 ± 0.1	70 (66–74)	32 (30–35)	0.5	6.5 ± 0.3
14	4130 ± 130	137 ± 4	30 ± 1	96 (89–104)	81 (78–89)	0.8	647 ± 24
15	9140 ± 260	290 ± 7	32 ± 1	154 (140–167)	267 (241–289)	1.7	805 ± 26
16	323 ± 6	20.4 ± 6	16 ± 1	41 (28–58)	58 (42–92)	1.4	60.1 ± 1.8
17	416 ± 7	30.6 ± 0.5	14 ± 1	234 (163–307)	296 (210–381)	1.3	50.9 ± 1.0
18	2290 ± 80	10.6 ± 0.5	220 ± 10	63 (49–81)	129 (91–196)	2.0	2170 ± 40
19	5240 ± 140	14.7 ± 0.3	360 ± 10	153 (109–211)	75 (53–111)	0.5	3110 ± 120
20^c	525 ± 32	9.9 ± 0.7	53 ± 5	91 (87–96)	332 (309–353)	4.0	188 ± 8
21^c	1360 ± 60	27.0 ± 0.8	50 ± 3	131 (120–145)	242 (186–316)	1.8	792 ± 40

^aSR = [k_i (AgAChE)]/[k_i (G119S AgAChE)].

^bRR = [LC₅₀(Akron)]/[LC₅₀(G3)].

^cThese k_i values were determined G3 and Akron homogenate as the source of WT and G119S AgAChE, respectively.

AChE inhibition IC₅₀ values (60 min incubation) for fluorinated methylketone inhibitors 22–27 and propoxur [68].

Table 5

Compound	AgAChE IC ₅₀ (nM)	G119S AgAChE IC ₅₀ (nM)	hAChE IC ₅₀ (nM)	SR ^a	S ^b
22	18.1 ± 0.4	20800 ± 1900	5.00 ± 0.16	1100 ± 100	0.3 ± 0.01
23	9.79 ± 0.32	996 ± 39	8.69 ± 0.18	100 ± 5	0.88 ± 0.03
24	953 ± 18	>10000	715 ± 15	>10	0.75 ± 0.02
25	0.68 ± 0.01	1730 ± 140	2.27 ± 0.04	2500 ± 200	3.3 ± 0.1
26	1.22 ± 0.03	25.1 ± 1.2 ^c	35.2 ± 0.7	21 ± 1	29 ± 1
27	337 ± 11	3000 ± 160	5190 ± 270	9 ± 1	15 ± 1
propoxur	43.6 ± 1.0	>10000	590 ± 19	>230	14 ± 1

^a Enzyme sensitivity ratio SR = [IC₅₀(G119S AgAChE)]/[IC₅₀(AgAChE)]

^b Selectivity (S) = [IC₅₀(hAChE)]/[IC₅₀(AgAChE)]

^c Compound 26 is a slow, tight-binding inhibitor of G119S AgAChE; value measured at steady state (23 h)