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Select β - and γ -branched 1-alkylpyrazol-4-yl methylcarbamates exhibit high selectivity for inhibition of *Anopheles gambiae* versus human acetylcholinesterase

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Abstract

The widespread emergence of pyrethroid-resistant *Anopheles gambiae* has intensified the need to find new contact mosquitocides for indoor residual spraying and insecticide treated nets. With the goal of developing new species-selective and resistance-breaking acetylcholinesterase (AChE)-inhibiting mosquitocides, in this report we revisit the effects of carbamate substitution on aryl carbamates, and variation of the 1-alkyl group on pyrazol-4-yl methylcarbamates. Compared to aryl methylcarbamates, aryl dimethylcarbamates were found to have lower selectivity for *An. gambiae* AChE (AgAChE) over human AChE (hAChE), but improved tarsal contact toxicity to G3 strain *An. gambiae*. Molecular modeling studies suggest the lower species-selectivity of the aryl dimethylcarbamates can be attributed to a less flexible acyl pocket in AgAChE relative to hAChE. The improved tarsal contact toxicity of the aryl dimethylcarbamates relative to the corresponding methylcarbamates is attributed to a range of complementary phenomena. With respect to the pyrazol-4-yl methylcarbamates, the previously observed low *An. gambiae*-selectivity of compounds bearing α -branched 1-alkyl groups was improved by employing β - and γ -branched 1-alkyl groups. Compounds **22a** (cyclopentylmethyl), **21a** (cyclobutylmethyl), and **26a** (3-

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methylbutyl) offer 250-fold, 120-fold, and 96-fold selectivity, respectively, for inhibition of *AgAChE* vs. *hAChE*. Molecular modeling studies suggests the high species-selectivity of these compounds can be attributed to the greater mobility of the W84 side chain in the choline-binding site of *AgAChE*, compared to that of W86 in *hAChE*. Compound **26a** has reasonable contact toxicity to G3 strain *An. gambiae* ($LC_{50} = 269 \mu\text{g/mL}$) and low cross-resistance to Akron strain ($LC_{50} = 948 \mu\text{g/mL}$), which bears the G119S resistance mutation.

Graphical Abstract

1. Introduction

Malaria is caused by infection with parasites of the genus *Plasmodium*, and is transmitted by Anopheline mosquitoes. It presents a terrible disease burden: in 2015 it is estimated to have killed 429,000 and sickened 212,000,000 people [1]. Of these deaths, 90% occurred in sub-Saharan Africa, and 70% of the deceased were children aged 5 years or less [1].

Remarkably, malaria mortality has been reduced 60% from 2000 to 2015 [1], and vector control methods have played an important role in this reduction of mortality. Specifically, the use of insecticide-treated bednets (ITNs) provide a direct physical and chemical barrier to disease transmission [2–5]. However, at present only pyrethroids are authorized by the World Health Organization for deployment on ITNs, and widespread pyrethroid resistance throughout Africa puts these huge gains at risk [6].

Pyrethroids target the voltage-gated sodium channel, so to circumvent resistance, ITN mosquitocides with different mechanisms of action are urgently needed.

Acetylcholinesterase (AChE) inhibition is a proven method to control adult malaria mosquitoes, but to date, anticholinesterases have never been approved for use on ITNs, and thus are used only for indoor residual spraying (IRS). These include the carbamates propoxur and bendiocarb, and organophosphates, in one case a longer lasting re-formulation of pyrimiphos- methyl [7]. The potential usefulness of an anticholinesterase for malaria control has prompted several groups to develop new AChE-targeting mosquitocides. Key challenges include high selectivity for inhibition of *Anopheles gambiae* AChE (*AgAChE*) vs human AChE (*hAChE*), and activity against the known resistance mutation (G119S) [8] with consequent high contact toxicity to adult G119S-*AgAChE*-bearing *An. gambiae*. These challenges have been met individually, but to date no single compound possesses both qualities.

For example, 2-substituted γ -branched aryl methylcarbamates **1a–3a** are quite selective (100- to 500-fold) for inhibition of wild type (WT) *AgAChE* vs *hAChE*, unlike 2-substituted α -branched aryl methylcarbamate propoxur **4a** (Figure 1) [9, 10].

Consistent with their inhibition of *AgAChE*, **1a–3a** are toxic to adult G3 strain *An. gambiae* in the tarsal contact assay, though not as toxic as **4a**. However, these compounds do not rapidly inactivate G119S *AgAChE*, and they have very low tarsal contact toxicity to Akron strain *An. gambiae*, which carry the G119S mutation of *AgAChE* [10]. Since these compounds target the catalytic serine of *AgAChE*, and since the G119S mutation was expected to reduce the volume of the active site, we explored carbamates derived from smaller 5-membered ring heterocycles. The 1-alkyl-pyrazol-4-yl methylcarbamate **5a** and 5-alkyl-isoxazol-3-yl dimethylcarbamates **6–8** proved to be excellent inhibitors of G119S *AgAChE* and were contact toxic to both G3 strain and Akron strain adult *An. gambiae* [11, 12]. However, these heterocyclic carbamates rapidly inactivated *hAChE*, and thus did not have the desired inhibition selectivity.

Thiol-reactive compounds **9** and **10** were designed by Pang and co-workers to target the free cysteine C286 in the acyl loop of *AgAChE*, which is a phenylalanine in *hAChE*, and these compounds and homologs offer exquisite selectivity for inhibition of *AgAChE* [13, 14]. However, to date, no insecticidal activity of these compounds has been disclosed. In 2012, Weill and co-workers disclosed a series of pyrimidinetrione analogs (e.g. **11**) that show preferential inhibition of G119S *Culex pipiens* AChE relative to the WT enzyme, and enhanced toxicity to *An. gambiae* larvae bearing that mutation [15]. However, the selectivity of these compounds vs *hAChE* is not known. Finally, Linusson and co-workers have recently disclosed two series of non-covalent inhibitors of *AgAChE*; compounds **12** and **13** show greater than 100-fold selectivity for inhibition of *AgAChE* vs *hAChE*. In addition, **13** and analogs were demonstrated to be toxic to susceptible adult *An. gambiae* [16, 17]. However, inhibitory potency of **13** against G119S *AgAChE* was poor [17].

Reviewing the data from **1a–5a** and **6–8**, the low inhibition selectivities exhibited by the β -branched isoxazol-3-yl dimethylcarbamate **7** (7-fold) and the γ -branched homolog **8** (2-fold) could be attributed to at least two phenomena. Firstly, it is possible that the putative selectivity pocket in *AgAChE* engaged by the γ -branched substituent of **1a–3a** cannot be reached by β -branched substituent of **7** or the γ -branched substituent of **8**, due to the different ring sizes and different placement of the alkyl groups relative to the carbamate in these two series. A second possibility is that the dimethylcarbamoyl functionality of **7–8** also serves to reduce inhibition selectivity. Note that these dimethylcarbamates were investigated as a matter of necessity, because the corresponding methylcarbamates were hydrolytically unstable [12].

To test these hypotheses, we prepared two series of compounds. We synthesized dimethylcarbamate and 1° carbamate derivatives of **1a–2a** and **4a–5a**, and reinvestigated the 1-alkyl pyrazol-4-yl methylcarbamate series of compounds, exploring analogs that possessed α -branched, β -branched, γ -branched, δ -branched, and unbranched alkyl groups. The overall goal of this study was to determine whether the appropriate 1-alkyl substituent on a pyrazol-4-yl methylcarbamate can confer excellent selectivity for inhibition of *AgAChE* vs

hAChE without reducing engagement of the G119S enzyme and toxicity to Akron strain *An. gambiae*.

2. Materials and methods

2.1 Insects

Susceptible G3 (MRA-112) and Akron strains (MRA-913) of *Anopheles gambiae* (African malaria mosquito) were obtained from BEI Resources through the CDC MR4 program. Akron mosquitoes have documented knockdown resistance (kdr) to pyrethroids (L1014F) and the G119S mutation (ace-1R), which confers resistance to carbamates. Mosquito colonies were maintained in the Department of Entomology, Virginia Tech, Blacksburg, VA 24061, USA, or raised up from eggs (obtained from CDC MR4, Atlanta, GA, USA) at the Emerging Pathogens Institute, University of Florida, Gainesville, FL 32610, USA. In all cases, mosquitoes were reared under 75% relative humidity and 28 °C, with a 12/12 dark and light cycle.

2.2 Chemicals

Structural information for the compounds assessed in this work are provided in Tables 1 and 2. Aryl methylcarbamates **1a**, **2a**, and **4a**, pyrazol-4-yl methylcarbamate **5a** and isoxaol-3-yl dimethylcarbamate **6** were prepared as described previously [9, 11, 12]. Aryl dimethylcarbamates **1b**, **2b**, **4b**, and pyrazol-4-yl dimethyl carbamate **5b** were prepared similarly by reaction of the corresponding phenol/pyrazol-4-ol precursors [9, 11] with *N,N*-dimethylcarbamoyl chloride. Primary carbamates **1c**, **2c**, **4c** and **5c** were prepared by reaction of the corresponding phenol/pyrazol-4-ol precursors [9, 11] with trichloromethylisocyanate (5 equiv), in dichloromethane; followed by passage through a neutral alumina column. Novel 1-alkyl-pyrazol-4-yl methyl carbamates **14a–33a** were prepared by deprotonation of the corresponding 1-alkyl-pyrazol-4-ols with potassium *t*-butoxide in THF/dichloromethane, followed by the addition of *N*-methylcarbamoyl chloride in dichloromethane, as previously described [11]. The requisite 1-alkyl-pyrazol-4-ols were prepared in three steps from pyrazole and the requisite alkyl halides or tosylates, as previously described [11]. All compounds were purified by column chromatography or preparative TLC on silica gel. Structural identity was confirmed in each case by NMR spectrometry (¹H, ¹³C), and high-resolution mass spectrometry. Greater than 95% purity was confirmed by ¹H NMR spectroscopy prior to biological assay.

2.3 Enzymes and Enzyme Inhibition Studies

Recombinant WT and G119S AgAChE were prepared as previously described [11]. Recombinant hAChE (C1682) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Inhibition potency of carbamate and carboxamide insecticides was assessed by measuring apparent second-order rate constants k_i (mM⁻¹ min⁻¹) for inactivation of the enzymes. We followed our previous progressive inactivation approach [9, 11, 12], in which enzymes were incubated with different concentrations of carbamates or carboxamides for differing times before measuring enzyme residual activity (v/v_0). Enzymes were incubated with typically five concentrations of inhibitors (and an inhibitor-free control) for up to 6 minutes at approximately 1 min intervals. Each concentration was present in the microplate in

duplicate, and each experiment was repeated. Note that for the G119S enzyme, inhibition was sometimes very low after 10 min at 10 μ M. In such cases, incubations with this enzyme were often extended to 60 min (10 min intervals). Residual activities v/v_0 are the ratio of the rate in the presence of inhibitor to a time-matched inhibitor-free control and thus correct for slow thermal inactivation of the enzyme. At each inhibitor concentration [I], plots of $\ln(v/v_0)$ vs. time (t) were constructed; the slopes of these unconstrained linear fits represent the pseudo first-order rate constants (k_{obs}) associated with those inhibitor concentrations. These k_{obs} values (obtained in at least two separate experiments) were then plotted against [I]; in each case the slope of the unconstrained linear fit is the apparent second-order rate constant k_i ($\text{mM}^{-1} \text{min}^{-1}$) for inactivation. Note that inhibitor concentrations [I] were chosen to be low enough to remain in the domain where a plot of k_{obs} vs [I] was linear. Enzymatic selectivity for inhibition was calculated from the measured k_i values; the error in these ratios was determined using a standard propagation of error method [18].

2.4 Tarsal contact toxicity assay

Adult female non-blood fed *An. gambiae* (both G3 and Akron strains) 3–5 days old, were used for filter paper assays of tarsal contact toxicity, which were performed in exposure tubes according to the 2006 World Health Organization recommendations [19] with slight modification. In brief, filter papers (15 x 12 cm) were treated with 2.0 mL of various concentrations of the carbamate in ethanol, and allowed to dry overnight. For the G3 strain, batches of 20–25 mosquitoes (in triplicate) were transferred to a holding tube and allowed to adapt for one hour. Due to lower colony numbers, toxicity assays with the Akron strain used batches of 10–15 mosquitoes in duplicate. Mosquitoes were then transferred to the exposure tube (held horizontally) that contained a treated filter paper. Knockdown was noted after 1 h, and all mosquitoes were transferred back to the holding tube (held upright), and given free access to 10% (w/v) sugar water. Mortality was recorded at 24 h. Both during exposure and the 23 h period following, mosquito tubes were kept in an environmental chamber at 24 ± 1 °C and 75% RH. To determine LC_{50} values, typically 5–8 concentrations were examined, and mortality data were used for probit analysis using PoloPlus [20] or SAS Probit.

3. Results

3.1 Inhibition selectivity and G3 strain *An. gambiae* tarsal contact toxicity of aryl and pyrazol-4-yl dimethylcarbamates

As can be seen in Table 1, addition of a methyl group to the carbamate nitrogen of *An. gambiae*-selective methylcarbamates **1a** (530-fold) and **2a** (130-fold) had a significant effect on the WT *AgAChE* inactivation rate constants (k_i), but less so for *hAChE*.

WT *AgAChE* k_i values of dimethylcarbamate derivatives **1b**, **2b** and **4b** were significantly reduced to 2–8% of their methylcarbamate analogs, but the corresponding *hAChE* k_i values were only reduced to 10–90% of those of the methylcarbamate analogs. Consequently, the selectivities of **1b**, **2b**, and **4b** were reduced to 20-, 59- and 1.5-fold respectively. Interestingly, for pyrazol-4-yl dimethylcarbamate **5b**, both the *AgAChE* and *hAChE* k_i values were reduced approximately 100-fold relative to the methylcarbamate **5a**. Thus the

low *AgAChE* selectivity of **5a** (7-fold) remained unchanged in **5b**. However, despite the significant decreases in *AgAChE* k_i values for the dimethylcarbamates, their G3 strain tarsal contact toxicities were not markedly reduced. Curiously, the dimethylcarbamates **1b** and **2b** were appreciably more contact toxic (~12-fold) than the methylcarbamates **1a** and **2a**. In the case of **4b** and **5b**, the toxicities were similar to their methylcarbamate analogs, despite the drastic decreases seen in *AgAChE* k_i values. Lastly, for G119S-*AgAChE*, the dimethylcarbamates **1b–2b**, **4b–5b** resembled the corresponding methylcarbamates in showing very slow inactivation.

3.2 Inhibition selectivity and G3 tarsal contact toxicity of 1° carbamates

Unsubstituted or 1° carbamates have been little explored as anticholinesterase insecticides since early studies by Metcalf [21], documented their lower insecticidal efficacy relative to the corresponding methylcarbamates. This report noted the hydrolytic instability of these compounds relative to the methylcarbamates, but we found that reproducible k_i values could be obtained with freshly prepared dilutions (Table 1). For **1c–2c** and **4c**, *AgAChE*, k_i values of the 1° carbamates were somewhat lower (40–70%) than those of the corresponding methylcarbamates. At *hAChE* k_i values of **1c–2c** and **4c** were very similar to those of the methylcarbamates. Thus, high selectivity was seen for **1c** (300-fold), but only moderate selectivity for **2c** (49-fold) and low selectivity for **4c** (13-fold). For **1c**, **2c**, and **4c**, no significant change was seen in k_i values at G119S-*AgAChE*. Interestingly, for the pyrazol-4-yl carbamate **5c**, increased k_i values were seen at all three enzymes. In fact, the rate constants measured for inactivation of WT *AgAChE* ($19,000 \text{ mM}^{-1} \text{ min}^{-1}$) and G119S-*AgAChE* ($587 \text{ mM}^{-1} \text{ min}^{-1}$) are the fastest we have measured thus far for any carbamate. However, the tarsal contact toxicity of these compounds to G3 strain of *An. gambiae* (Table 1) ranged from very low for **1c** & **2c** (no mortality at 1000 $\mu\text{g/mL}$) to moderate for **4c** (LC_{50} ~500 $\mu\text{g/mL}$) to excellent for **5c** (LC_{50} = 90 $\mu\text{g/mL}$, equitoxic to **5a**).

3.3 Inhibition selectivity and G3 tarsal contact toxicity of β -branched and γ -branched 1-alkyl-pyrazol-4-yl methylcarbamates

The comparative study of aryl methyl and dimethylcarbamates above confirmed that dimethylcarbamates can have reduced selectivity for inhibition of WT *AgAChE*, providing a possible explanation for the low species-selectivity of 5-alkyl-isoxazol-3-yl dimethylcarbamates **7** and **8**. Accordingly, a collection of pyrazol-4-yl methylcarbamates bearing α -branched, β -branched, γ -branched, δ -branched and unbranched 1-alkyl groups were prepared and assayed for enzymatic selectivity (Table 2). For *hAChE*, the highest k_i values were seen for the α -branched compounds (**5a**, **14a–18a**, $80\text{--}805 \text{ mM}^{-1} \text{ min}^{-1}$). Corresponding k_i values for β -branched (**19a–25a**, $0.5\text{--}13 \text{ mM}^{-1} \text{ min}^{-1}$), γ -branched (**26a–30a**, $0.6\text{--}28 \text{ mM}^{-1} \text{ min}^{-1}$), δ -branched (**31a–32a**, $3\text{--}4 \text{ mM}^{-1} \text{ min}^{-1}$) and unbranched (**33a**, $28 \text{ mM}^{-1} \text{ min}^{-1}$) compounds were considerably smaller.

In aggregate, compounds bearing α -branched 1-alkyl groups also showed the highest WT *AgAChE* k_i values ($500\text{--}9,000 \text{ mM}^{-1} \text{ min}^{-1}$), but k_i values for β -branched ($5\text{--}1,500 \text{ mM}^{-1} \text{ min}^{-1}$) and γ -branched compounds ($8\text{--}1,770 \text{ mM}^{-1} \text{ min}^{-1}$) were not as diminished as they were in the case of *hAChE*. Consequently, β - and γ -branched compounds showed higher selectivity for inhibition of WT *AgAChE* than did the α -branched compounds. Compounds

21a (120-fold selective) and **22a** (250-fold selective) are β -branched, bearing cyclobutylmethyl and cyclopentylmethyl groups respectively. Compounds **26a** (96-fold) and **27a** (63-fold) are γ -branched, bearing 3-methylbutyl and 2-cyclopropylethyl groups, respectively. Regarding G119S *AgAChE* k_i values, we would note that the magnitudes and trends seen in hAChE k_i values are maintained. α -Branched compound k_i values (**14a–18a**, 11–290 mM⁻¹ min⁻¹) were larger than those for β -branched (**19a–25a**, 0.5–14 mM⁻¹ min⁻¹), γ -branched (**26a–30a**, 0.5–23 mM⁻¹ min⁻¹), δ -branched (**31a–32a**, ~2 mM⁻¹ min⁻¹) and unbranched (**33a**, 18 mM⁻¹ min⁻¹) compounds.

The tarsal contact toxicity data in Table 2 against G3 and Akron strain *An. gambiae* showed that, in general, the α -branched compounds (**14a–18a**) have the greatest toxicity, with cross resistance in the Akron strain of less than 2-fold (Table 2). Exceptions were compounds **17a** (12.6-fold cross resistance) and **19a**, which matched the enzyme inhibition potency and lethality to G3 females of **4a** with cross resistance in Akron of 5.6-fold (Table 2).

Unfortunately, toxicities of the species-selective carbamates bearing β - and γ -branched alkyl groups (**21a**, **22a**, **26a**, **27a**) were markedly reduced. Yet, one selective compound **26a** (96-fold selectivity) had a G3 strain tarsal contact LC₅₀ value of 269 μ g/mL, 5 to 6-fold lower than that of the selective aryl methylcarbamates **1a** and **2a**, respectively (c.f. Table 1).

Furthermore, unlike **1a** and **2a**, compound **26a** rapidly inactivates G119S *AgAChE* (k_i = 5.88 mM⁻¹ min⁻¹), and shows low cross-resistance to Akron strain (LC₅₀ = 948 μ g/mL) of 3.5-fold (Table 2).

4. Discussion

As mentioned previously, in all cases examined thus far, dimethylcarbamates inactivate WT *AgAChE* much more slowly than the corresponding methylcarbamates (Table 1, cf. **1a**, **1b**; **2a**, **2b**; **4a**, **4b**; **5a**, **5b**). A decreased rate is also observed for hAChE, as might be expected on the basis of greater steric hindrance of the dimethylcarbamate carbonyl group to nucleophilic attack by the catalytic serine. Yet, dimethylcarbamates show less diminishment in hAChE k_i values compared to *AgAChE* k_i values, suggesting that the acyl pocket of hAChE is somehow more accommodating to the additional methyl group. To understand this trend, we examined the 2.2 Å structure (PDB ID: 1GQR) [22] of the ethylmethylcarbamoyl form of *Torpedo californica* AChE (*TcAChE*), resulting from its covalent inhibition by rivastigmine (Fig. 2, gray sticks in Fig. 2B).

On this structure is superimposed the apo form of hAChE (green sticks, PDB ID: 4EY4, 2.16 Å)[23] and the recent apo G119S *AgAChE* structure (magenta sticks, PDB ID: 6ARX, 2.3 Å).[24] The ethylmethylcarbamoyl group (gray spheres) is bound to the catalytic serine (S200 in *TcAChE*), and projects into the acyl pocket, which is largely defined by conserved sidechains (*Tc/Ag/h* respectively: W233/232/236, F290/288/297 and F331/329/338, not shown in Figure 2B). However, as Figure 2B illustrates, the side of the acyl pocket closest to the CH₂ unit of the ethylmethylcarbamoyl group is delimited by a phenylalanine sidechain that comes from a different part of the enzyme fold in *TcAChE*/hAChE vs. *AgAChE*. In *TcAChE* and hAChE, the sidechain of F288/295 is in a relatively mobile loop, as suggested by the movement of the F288 sidechain seen in ethylmethylcarbamoyl *TcAChE* (gray) relative to apo hAChE (green). In contrast, the delimiting phenylalanine (F399) in *AgAChE*

belongs to a deeply buried end of a helix that is likely quite static, and appears too close to the CH₂ group of rivastigmine. Thus, we attribute greater expansion plasticity to the acyl pocket of hAChE, allowing adaptation to variously sized moieties. This flexibility reduces the impact of the additional methyl group on hAChE k_i values relative to the AgAChE k_i values, thus reducing Ag/h inhibition selectivity (Table 1).

Another feature of the dimethylcarbamates that deserves commentary is their greater than expected tarsal contact toxicity, given their low WT AgAChE k_i values relative to the corresponding methylcarbamates. For example, **1b** and **2b** are 10-fold more toxic than **1a** and **2a**, despite the fact that their WT AgAChE k_i values are decreased by more than 95% relative to **1a** and **2a**. Metabolism is a likely contributing factor, and dimethylcarbamates are known to undergo oxidative metabolism to the methylcarbamates [25], which are more potent inhibitors (Table 1). In contrast, methylcarbamates are expected to undergo oxidative metabolism to the 1° carbamates, which are poorer inhibitors, except in the case of **5c** and **5a** (Table 1). In this connection, it is interesting to note that 1° carbamates **1c**, **2c**, and **4c**, are less toxic than their methylcarbamate analogs (**1a**, **2a**, **4a**), but that 1° carbamate **5c** is equitoxic to **5a**. However, preliminary piperonyl butoxide synergism studies (data not shown) did not show major effects (i.e. >2-fold) on the toxicity of the methylcarbamates or dimethylcarbamates. For this reason, we propose that additional effects contribute to the high tarsal contact toxicity of dimethylcarbamates. Firstly, decreased rates of hydrolysis of dimethylcarbamates to the phenols, relative to methylcarbamate and 1° carbamates, may contribute to the higher concentrations of the dimethylcarbamates in the CNS following tarsal contact. Secondly, the greater lipophilicity of dimethylcarbamates relative to methylcarbamates (cf. CLogP values of 1.69 and 1.48 for **4b** and **4a**) may lead to improved transfer efficiency from paper to the mosquito tarsi, and improved transport through the cuticle to the CNS. Further experimentation would be required to test these hypotheses.

With regard to the studies of pyrazol-4-yl methylcarbamates, hAChE and WT AgAChE inactivation rate constants (k_i) are significantly affected by the location of the branch in the 1-alkyl group. Compounds bearing α -branched alkyl groups give the highest inactivation rates at WT AgAChE and hAChE, suggesting favorable hydrophobic interactions of the 1-alkyl groups with a pocket close to the catalytic serine in both enzymes. As the branch point is moved from the α -carbon to the β - or γ -carbon, the hAChE k_i values are reduced significantly more than are the corresponding WT AgAChE k_i values, suggesting that this hydrophobic pocket is more flexible in WT AgAChE than in hAChE. Note that this trend is seen within three isomeric series (Figure 3: a) **5a**, **19a**, **33a**; b) **17a**, **21a**, **27a**; c) **15a**, **26a**), thus minimizing the likelihood that the trends in k_i values are affected by variations in molecular weight or CLogP.

The recent X-ray structure of the covalent complex of G119S AgAChE in complex with the potent 1-alkyl-pyrazol-4-yl difluoromethyl ketone inhibitor **34** [24, 26] does provide support for the aforementioned proposal that the 1-alkyl groups of the pyrazol-4-yl methylcarbamates project into a hydrophobic pocket that is more flexible in AgAChE than in hAChE. In this structure (PDB ID: 6ARY, not shown), the α -branched 3-pentyl group of **34** (see Fig. 4A) projects into a hydrophobic pocket partially defined by the choline-binding site tryptophan W84 [24]. On this basis, we propose that the alkyl groups of 1-alkyl-

pyrazol-4-yl methylcarbamates (**5a**, **14a–33a**) project into the same region in their tetrahedral adducts with catalytic serine. We further note that in this X-ray structure of **34** complexed to G119S *AgAChE* (PDB ID: 6ARY) the 3-pentyl moiety of **34** already fills nearly all the available space between the pyrazole ring and the indole of W84.

Based on that structure, it is not clear how *AgAChE* could accommodate larger groups such as the cyclopentylmethyl group of selective inhibitor **22a**, unless the indole unit moved. In *hAChE*, the position of the indole side chain of W86 is locked by hydrogen-bonding from Y449 (gray sticks, Fig. 4B).

Although the indole side chain of W84 in G119S *AgAChE* also adopts this canonical orientation (magenta sticks), we propose the replacement of Y449 (*hAChE*) by D441 (*AgAChE*) renders the W84 side chain more mobile, since the stabilizing hydrogen bond is lost. Our preliminary modeling of G119S *AgAChE* identified several low energy rotameric states of W84, including the one depicted in aquamarine sticks in Fig. 4B. In this alternate conformation, the hydrophobic pocket of *AgAChE* can just accommodate the β -branched methylcyclopentyl group of **22a**, but not the larger methylcyclohexyl and $\text{CH}_2\text{CH}(\text{Et})_2$ groups of **23a** and **24a** respectively (Fig. 4C). In contrast, the smaller hydrophobic pocket in *hAChE* defined by the canonical orientation of W86 cannot well accommodate **22a**. This model thus provides an explanation for the high *Ag/h* selectivity of **22a**, and the lower selectivities of **23a** and **24a**.

Lastly, as we have noted in our discussion of the dimethylcarbamates above, and in previous studies [10, 12], tarsal contact toxicity of carbamates remains a complex function of several physiochemical and pharmacokinetic parameters, not only *AgAChE* inhibitory potency. Within each isomeric series, molecular weight and topological polar surface area (tPSA) are identical, and CLogP varies only slightly. Yet within these three series there is no correlation of *AgAChE* k_i values to G3 strain LC₅₀ values, pointing to structure-dependent metabolism and active transport as modulators of toxicity.

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Highlights

- Aryl and pyrazol-4-yl carbamates were explored as *An. gambiae* and human AChE inhibitors
- Dimethylcarbamates have lower inhibition selectivity than methylcarbamates
- Beta- and gamma-branched alkyl groups confer *An. gambiae* AChE inhibition selectivity
- Comparison of hAChE and G119S AgAChE crystal structures explains these selectivities

Fig. 1. Representative compounds investigated as *An. gambiae*-selective (**1a–3a, 9,10,12,13**) or resistance-breaking (**5a, 6–8, 11**) acetylcholinesterase inhibitors. Propoxur (**4a**) is approved for IRS but has modest selectivity for *An. gambiae* [9] and is not resistance-breaking [11].

Fig. 2.

A) Comparison of the structures of rivastigmine, dimethylcarbamate **4b**, and methylcarbamate **4a**. The transferrable ethylmethylcarbamoyl portion of rivastigmine is shown in bold blue; its CH₂ unit is indicated with an arrow. B) Overlay of select residues in ethylmethylcarbamoyl *T. californica* AChE (gray PDB ID: 1GQR), apo hAChE (green, PDB ID: 4EY4, 2.16 Å) and apo G119S *AgAChE* (magenta, PDB ID: 6ARX, 2.30 Å). The ethylmethylcarbamoyl group of rivastigmine (gray spheres) is covalently attached to the catalytic serine of *TcAChE* (S200); the phenolic leaving group is not shown. Note that *AgAChE* residue numberings begin with the start of the conserved catalytic subunit, and do not include the 161-amino acid N-terminal domain. Thus, D1 is D162 in full-length *AgAChE*, and F399 is F560 in full-length *AgAChE*.

	α -branched	β -branched	γ -branched	unbranched
a) C_6H_9 -substituted			none possible	
	5a CLogP: 0.693	19a CLogP: 0.783		33a CLogP: 0.913
hAChE k_i ($\text{mM}^{-1} \text{min}^{-1}$)	647 $\pm$ 24	6.51 $\pm$ 0.35		27.8 $\pm$ 1.3
AgAChE k_i ($\text{mM}^{-1} \text{min}^{-1}$)	4,130 $\pm$ 130	280 $\pm$ 5		1,070 $\pm$ 10
Ag/h selectivity	6.4 $\pm$ 0.3	43 $\pm$ 2		38 $\pm$ 2
G3 strain LC_{50} ($\mu\text{g/mL}$)	96 (89-104)	41 (38-44)		<1,000
b) C_6H_9 -substituted				not prepared
	17a CLogP: 0.798	21a CLogP: 0.858	27a CLogP: 0.828	
hAChE k_i ($\text{mM}^{-1} \text{min}^{-1}$)	174 $\pm$ 7	13.1 $\pm$ 0.5	28.1 $\pm$ 1.0	
AgAChE k_i ($\text{mM}^{-1} \text{min}^{-1}$)	2,380 $\pm$ 50	1,520 $\pm$ 30	1,770 $\pm$ 30	
Ag/h selectivity	14 $\pm$ 1	120 $\pm$ 10	63 $\pm$ 3	
G3 strain LC_{50} ($\mu\text{g/mL}$)	29 (26-32)	<1,000	>1,000	
c) C_5H_{11} -substituted		not prepared		not prepared
	15a CLogP: 1.222		26a CLogP: 1.312	
hAChE k_i ($\text{mM}^{-1} \text{min}^{-1}$)	805 $\pm$ 36		4.87 $\pm$ 0.29	
AgAChE k_i ($\text{mM}^{-1} \text{min}^{-1}$)	9,140 $\pm$ 260		468 $\pm$ 21	
Ag/h selectivity	11 $\pm$ 1		97 $\pm$ 7	
G3 strain LC_{50} ($\mu\text{g/mL}$)	154 (140-167)		269 (253-283)	

Fig. 3. Comparison of hAChE k_i , AgAChE k_i , Ag/h inhibition selectivity, and G3 strain tarsal contact toxicity within three isomeric series a)–c), varying the branch point of the 1-alkyl group from α - to β - to γ -branched to unbranched. Data are taken from Table 2. Note there is minimal variation in CLogP within each isomeric series; calculated topological polar surface area (tPSA) is 53.9 Å² for all structures (ChemDraw Professional 16.0).

Fig. 4.
A) Covalent difluoromethyl ketone inhibitor **34**, and *An. gambiae*-selective inhibitor **22a**. B) Superposition of the choline-binding sites of hAChE (PDB ID: 1EY4, gray sticks), and apo G119S AgAChE (PDB ID: 6ARX, magenta ribbon, magenta sticks). Note the canonical orientation of the indole rings of W86 (human) and W84 (apo G119S AgAChE). An alternate conformation of the indole of W84 is shown in aquamarine sticks; this movement expands the hydrophobic binding pocket of AgAChE. C) A view of the tetrahedral intermediate of AgAChE-selective inhibitor **22a** with G119S AgAChE, focusing on the cyclopentyl ring of the inhibitor. The surface of the hydrophobic binding pocket of AgAChE is colored gray, except for the surface defined by the indole ring of the W84 in its alternate conformation, which is painted aquamarine. Note that AgAChE residue numberings begin with the start of the conserved catalytic subunit, and do not include the 161-amino acid N-terminal domain. Thus, W84 is W245 in full-length AgAChE.

Enzyme inhibition and tarsal contact toxicity of methylcarbamates (**1a–2a**, **4a–5a**), dimethylcarbamates (**1b–2b**, **4b–5b**, **6**), and 1° carbamates (**1c–2c**, **4c–5c**).

Table 1

Compound ^a	k_i (mM ⁻¹ min ⁻¹) ^b					G3 LC ₅₀ (μg/mL) ^d
	hAChE	WT AgAChE	G119S AgAChE	WT Ag/h selectivity ^c		
1a [9, 10]	0.48 ± 0.11 ^e	255 ± 12 ^e	<0.060 ± 0.015 ^e	530 ± 130 ^e		1,388 (1,020–1,751)
1b	0.19 ± 0.02	3.73 ± 0.13	0.056 ± 0.15	20 ± 2		111 (58–177)
1c	0.416 ± 0.032	127 ± 5	0.01 ± 0.01	310 ± 26		> 1,000 ^f
2a [9, 10]	14.5 ± 1.5 ^e	1,850 ± 100 ^e	<0.047 ± 0.019 ^e	130 ± 15 ^e		1,736 (1,120–2,292)
2b	1.45 ± 0.06	85.7 ± 5.5	0.040 ± 0.008	59 ± 5		140 (73–233)
2c	14.6 ± 0.7	713 ± 19	0.01 ± 0.01	49 ± 3		> 1,000 ^f
4a [9, 10] (propoxur)	17.0 ± 0.4 ^e	266 ± 9 ^e	<0.037 ± 0.007 ^e	16 ± 1 ^e		39 (32–45) ^e
4b	14.8 ± 0.5	22.1 ± 0.5	<0.025 ± 0.011	1.5 ± 0.1		59 (44–87)
4c	13.9 ± 0.7	185 ± 5	0.01 ± 0.01	13 ± 1		~500 ^g
5a [11]	647 ± 24 ^e	4,130 ± 130 ^e	137 ± 4 ^e	6.4 ± 0.3 ^e		96 (89–104) ^e
5b	5.28 ± 0.59	36.7 ± 0.8	2.27 ± 0.24	7 ± 1		124 (117–132)
5c	1,520 ± 130	19,000 ± 600	587 ± 37	12 ± 1		90 (79–100)
6 [12]	60.1 ± 1.8 ^e	323 ± 6 ^e	20.4 ± 0.6 ^e	5.4 ± 0.2 ^e		41 (28–58) ^e

^aCompounds **1a**, **2a**, **4a**, **5a**, **6** were reported previously.

^b Apparent bimolecular rate constants for enzyme inactivation, measured as described in Section 2.3 above.

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^c Selectivity ratio is calculated as $k_i(\text{WT})/k_i(\text{hACHE})$, with standard error in the ratio calculated according to propagation of error (see Section 2.3 above).[18]

^d LC₅₀ values are the concentration of inhibitor used to treat the paper that killed 50% of the insects in the tarsal contact assay (see Section 2.3 above).

^e Data reported previously.

^f No mortality at this concentration.

^g 100% mortality @ 1,000 µg/mL and 0% mortality @ 100 µg/mL

Enzyme inhibition and tarsal contact toxicity of 1-alkylpyrazol-4-yl methylcarbamates **5a**, **14a–33a** bearing various N1-substituents (R); the structures of *An. gambiae*-selective compounds **21a**, **22a**, **26a**, and **27a** are highlighted for reference.

Table 2

Compound ^a	R	k_i (mM ⁻¹ min ⁻¹) ^b			LC ₅₀ (μg/mL) or % mortality at 1000 μg/mL	
		hAChE	WT AgAChE	G119S AgAChE	Ag/h selectivity ^c	Akron strain
5a	<i>s</i> -Bu	647 ± 24	4,130 ± 130	137 ± 4	6.4 ± 0.3	81 (78–89)
14a	<i>i</i> -Pr	79.8 ± 2.4	498 ± 13	10.7 ± 0.1	6.2 ± 2	650 (488–859)
15a	2-pentyl	805 ± 36	9,140 ± 260	290 ± 7	11 ± 1	267 (241–289)
16a	3-pentyl	168 ± 8	2,220 ± 70	125 ± 3	13 ± 1	231 (217–245)
17a	<i>c</i> -C ₅ H ₉	174 ± 7	2,380 ± 50	36.5 ± 0.8	14 ± 1	365 (344–384)
18a	2-heptyl	585 ± 29	2,620 ± 60	51.4 ± 3.6	4.5 ± 0.2	40%
19a	<i>i</i> -Bu	6.51 ± 0.35	280 ± 5	4.51 ± 0.12	43 ± 2	228 (181–286)
20a	-CH ₂ <i>c</i> -C ₃ H ₅	0.53 ± 0.05	20.3 ± 0.6	1.04 ± 0.04	38 ± 4	ND
21a	-CH ₂ <i>c</i> -C ₄ H ₇	13.1 ± 0.5	1,520 ± 30	13.5 ± 0.4	120 ± 10	10%
22a	-CH ₂ <i>c</i> -C ₅ H ₉	1.93 ± 0.24	477 ± 38	6.16 ± 0.24	250 ± 40	0%
23a	-CH ₂ <i>c</i> -C ₆ H ₁₁	0.93 ± 0.03	10.8 ± 0.5	0.69 ± 0.02	12 ± 1	17%
24a	-CH ₂ CH(C ₂ H ₅) ₂	6.98 ± 0.27	33.7 ± 1.0	1.15 ± 0.11	4.8 ± 2	ND
25a	-CH ₂ Ph	1.29 ± 0.06	4.74 ± 0.21	0.55 ± 0.05	3.7 ± 0.2	0%
26a	-CH ₂ CH ₂ CH ₂ -Me ₂	4.87 ± 0.29	468 ± 21	5.88 ± 0.21	96 ± 7	269 (253–283)
27a	-CH ₂ CH ₂ <i>c</i> -C ₃ H ₅	28.1 ± 1.0	1,770 ± 30	22.8 ± 1.2	63 ± 3	10%
28a	-CH ₂ CH ₂ <i>c</i> -C ₄ H ₇	15.8 ± 1.1	463 ± 14	9.64 ± 0.45	29 ± 2	ND
29a	-CH ₂ CH ₂ <i>c</i> -C ₅ H ₉	8.66 ± 0.43	25.1 ± 1.0	2.58 ± 0.12	2.9 ± 0.2	ND
30a	-CH ₂ CH ₂ CH ₂ -Me(OMe)	0.60 ± 0.04	8.12 ± 0.18	0.52 ± 0.06	14 ± 1	45%

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Compound ^a	R	k_i (mM ⁻¹ min ⁻¹) ^b					LC ₅₀ (μg/mL) or % mortality at 1000 μg/mL	
		hAChE	WT AgAChE	G119S AgAChE	Ag/h selectivity ^c	G3 strain	Akron strain	
31a	CH ₂ CH ₂ CH ₂ -CHMe ₂	4.33 ± 0.15	25.7 ± 1.3	1.91 ± 0.05	5.9 ± 0.4	10%	ND	
32a	-CH ₂ CH ₂ CH ₂ Ph	3.40 ± 0.01	18.8 ± 0.6	1.80 ± 0.13	5.5 ± 0.2	10%	ND	
33a	Bu	27.8 ± 1.3	1,070 ± 10	18.2 ± 0.7	38 ± 2	100%	16%	

^aData for **5a**, **14a–17a** were reported previously [11].

^b Apparent bimolecular rate constants for enzyme inactivation (mM⁻¹ min⁻¹), as described previously.[10, 12]

^c Selectivity is calculated as $k_i(\text{WT AgAChE})/k_i(\text{hAChE})$, with standard error in the ratio calculated according to standard propagation of error.[18]

^d LC₅₀ values are the concentration of inhibitor used to treat the paper that killed 50% of the insects in the tarsal contact assay (see Section 2.3 above). ND designates ‘not determined’; compounds that did not show significant toxicity to susceptible G3 strain were generally not tested on the resistant Akron strain.