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Towards a species-selective acetylcholinesterase inhibitor to control the mosquito vector of malaria, *Anopheles gambiae*

Paul R. Carlier[a,*], Troy D. Anderson[b], Dawn M. Wong[a], Danny C. Hsu[a], Joshua Hartsel[a], Ming Ma[a], Eric A. Wong[c], Ranginee Choudhury[c], Polo C.-H. Lam[d], Maxim M. Totrov[d], Jeffrey R. Bloomquist[b]

[a] *Department of Chemistry, Virginia Tech, Blacksburg, VA 24061, USA*
[b] *Department of Entomology, Virginia Tech, Blacksburg, VA 24061, USA*
[c] *Department of Animal and Poultry Sciences, Virginia Tech, Blacksburg, VA 24061, USA*
[d] *Molsoft LLC, 3366 North Torrey Pines Court, Suite 300, La Jolla, CA 92037, USA*

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ABSTRACT

Anopheles gambiae is the major mosquito vector of malaria in sub-Saharan Africa. At present, insecticide-treated nets (ITNs) impregnated with pyrethroid insecticides are widely used in malaria-endemic regions to reduce infection; however the emergence of pyrethroid-resistant mosquitoes has significantly reduced the effectiveness of the pyrethroid ITNs. An acetylcholinesterase (AChE) inhibitor that is potent for *An. gambiae* but weakly potent for the human enzyme could potentially be safely deployed on a new class of ITNs. In this paper we provide a preliminary pharmacological characterization of *An. gambiae* AChE, discuss structural features of *An. gambiae* and human AChE that could lead to selective inhibition, and describe compounds with 130-fold selectivity for inhibition of *An. gambiae* AChE relative to human AChE.

1. Introduction

Acetylcholinesterase (AChE: EC 3.1.1.7) catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh) and is primarily responsible for termination of cholinergic neurotransmission at synapses in the central nervous system of both humans and insects. Significant inhibition of AChE is lethal and thus AChE-directed insecticides have received tremendous attention over the last 50 years [1]. Because human toxicity resulting from concurrent human AChE (*h*AChE) inhibition remains a significant problem [2], we seek to develop new insecticidal AChE inhibitors that possess exceptional target selectivity for *Anopheles gambiae* (*An. gambiae*), the mosquito vector of malaria. If such compounds could be identified, they might be ideally suited for deployment on insecticide-treated nets, which provide the first line of defense against malaria infection in sub-Saharan Africa [3]. However to date, very little structure–activity data on inhibitors of *An. gambiae* AChE (*Ag*AChE) have appeared in the public literature.

Since publication of the first X-ray crystal structure of AChE in 1991 [4], a wealth of high resolution structural information has become available that could inform the design of an *Ag*AChE-selective inhibitor. A search of the Protein Data Bank [5], Esther [6], and NCBI Structure databases revealed that 96 crystal structures of AChE of better than 3.2 Å resolution were publicly available as of October 2007. However, to date only one insect species of AChE has been crystallized, that of *Drosophila melanogaster* (*Dm*AChE, three structures: PDB ID 1qo9, 1qon, 1dx4) [7]. It should be noted that a homology model of *Ag*AChE was developed to explain the effect of the known G119S resistance mutation

* Corresponding author at: Department of Chemistry, Virginia Tech, Hahn Hall 3103, West Campus Drive, Blacksburg, VA 24061, USA.
Tel.: +1 540 231 9219; fax: +1 540 231 3255.
E-mail address: pcarlier@vt.edu (P.R. Carlier).

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	catalytic triad			oxyanion hole		choline-binding site	
	200	327	440	116	201	84	330
Tc	TIFGE**S**AGGAS	**E**	**H**	YG**G**G**F**	**A**	W	FF
	203	334	447	119	204	86	337
human	TLFGE**S**AGAAS	**E**	**H**	YG**G**G**F**	**A**	W	YF
	199	325	439	116	200	84	328
Ag ace-1	TLFGE**S**AGAVS	**E**	**H**	FG**G**G**F**	**A**	W	YF
	238	367	480	148	239	83	370
Dm ace-2	TLFGE**S**AGSSS	**E**	**H**	YG**G**G**F**	**A**	W	YF

	acyl pocket			peripheral site					flexible peripheral site loop	
	233	288	290	70	72	121	279	334	279	291
Tc	W	F	F	Y	D	Y	W	YG	WNVLPFDSIFRFS	
	236	295	297	72	74	124	286	341	286	298
human	W	F	F	Y	D	Y	W	YG	WHVLPQESVFRFS	
	232	286	288	70	72	121	280	332	280	289
Ag ace-1	W	C	F	I	D	Y	W	YY	WGTL---GICEFP	
	271	328	330	69	71	153	321	374	321	331
Dm ace-2	W	L	F	E	Y	M	W	YD	WNSY--SGILSFP	

Fig. 1. Alignment of *Torpedo californica*, human, *Anopheles gambiae* (*ace*-1), and *Drosophila melanogaster* (*ace*-2) AChE. SwissProt codes: ACES_TORCA (*Tc*); ACES_HUMAN (human); ACES_ANOGA (*Ag ace*-1); ACES_DROME (Dm ace-2). Residues marked in bold are essential for catalysis. By convention, numbering is based on that of the catalytic subunit/mature form of *Tc*AChE, as defined by X-ray structures of the protein (e.g., PDB ID 2ace).

[8], and a computationally refined homology model [9] has been publicly deposited in the Protein Data Bank (PDB ID 2AZG).

Surprisingly, *Dm*AChE shares only 41% sequence identity (56% similarity) with *Ag*AChE, perhaps due to the fact that the major ACh-hydrolyzing enzymes of these two species are encoded by different genes. *An. gambiae* is one of several insect species known to carry two AChE genes (*ace*-1 and *ace*-2), but a range of compelling evidence has established that *Ag*AChE is encoded by *ace*-1 [10]. In contrast, *D. melanogaster* carries a single non-homologous gene (*ace*-2) to encode *Dm*AChE [10]. Although the sequence identity of *Ag*AChE compared to human AChE (*h*AChE) is marginally higher (49%, with 65% similarity) than that with *Dm*AChE, the overall low sequence identity to *h*AChE suggests that it may be possible to develop AChE inhibitors with high *Ag*AChE/*h*AChE selectivity. Radić and Taylor have recently published an excellent large-scale alignment of 125 AChE sequences [11]; similar multiple sequence alignments have also recently been published by Pezzementi et al. [12] and Pang [9]. Fig. 1 presents portions of the alignment of *Torpedo californica* AChE (*Tc*AChE), *h*AChE, *Ag*AChE, and *Dm*AChE. Six key regions of the enzyme are selected for comparison: the catalytic triad, the oxyanion hole, the choline-binding site, the acyl pocket, the peripheral site, and the "flexible peripheral site loop." This latter region has previously been referred to as the "acyl pocket loop" [11,13], and in *Tc*AChE as the "W279-S291 loop," where its flexibility has been noted [14–17]. For ease of reference, Fig. 1 also provides the appropriate *h*, *Ag*, and *Dm* residue numberings to accompany the conventional *Tc*AChE numberings.

As might be expected, identity between human and *Ag ace*-1 is extremely high throughout the catalytic triad region, the oxyanion hole, and the choline-binding site. However, interesting and potentially useful differences are seen in the acyl pocket, the peripheral site, and the flexible peripheral site loop; these are discussed further below. Finally, the significant difference in residue numberings between *Dm* and the other species following *Tc* residue 104 (not shown in Fig. 1) is a consequence of the so-called "hydrophilic insertion" of 31 amino acids, which appears characteristic of the *ace*-2 gene, at least in diptera [10].

2. Methods and materials

2.1. Inhibitors

Except for ethopropazine, bis(7)-tacrine, iodoacetamides **2a**–**h**, and carbamates **4a**–**d**, all inhibitors were purchased. Ethopropazine was prepared by alkylation of phenothiazine with 1-chloro-*N*,*N*-diethylpropan-2-amine [18]. Bis(7)-tacrine was prepared by the literature method [19]; the iodoacetamides were prepared by acylation of the known 9-(ω-aminoalkylene)-tacrines **1a**–**h** [20], as described below in Scheme 1. Carbamates **4a**–**d** were prepared by *N*-methylcarbamoylation of the corresponding phenols, which were commercial or synthesized [21]. All compounds were >95% pure by ^{1}H NMR analysis and gave correct ^{13}C NMR and mass spectra.

2.2. Sequence alignment

Alignments of *Tc*AChE, *h*AChE, *Ag*AChE, and *Dm*AChE were performed using CLUSTAL W (1.83) multiple sequence alignment (http://www.ebi.ac.uk/) [22] on the full-length precursor sequences (SwissProt codes ACES_TORCA, ACES_HUMAN, ACES_ANOGA, and ACES_DROME, respectively). Alignments were then cross-checked against the published, crystallized mature forms of *Tc*AChE (PDB ID 2ace) [23], *h*AChE (PDB ID 1b41) [24], and *Dm*AChE (PDB ID 1dx4) [7]. Overlay of the *Ag*AChE homology model (see below) with *Tc*AChE prompted manual alignment of