Pesticide Biochemistry and Physiology 106 (2013) 85–92

Contents lists available at SciVerse ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

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

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ARTICLE INFO

Article history:
Available online 28 March 2013

Keywords:
Leishmaniasis
Sandfly
Cattle fever
Southern cattle tick
Insecticides
Selectivity

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* (*Bm*AChE1) and *Pp* (*Pp*AChE) compared to human and bovine AChE, in order to identify divergent pharmacology that might lead to selective inhibitors. Results indicate that *Bm*AChE has low sensitivity (IC_{50} = 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 *Bm*AChE. Molecular homology models suggest the r*Bm*AChE 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, *Bm*AChE1 and *Pp*AChE 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 *Bm*AChE1 and *Pp*AChE over human AChE, and a mouse oral LD_{50} of >1500 mg/kg, thus providing an excellent new lead for vector control.

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 potentially deadly pest of cattle, since it is a primary vector for babesiosis and anaplasmosis [2]. Economic losses are furthered substantially as normal feeding behavior of tick infestations lead to reduction in milk production and weight gain, as well as overall declines in cattle health [3]. Similarly, the sandfly, *Pp* is a primary vector of numerous zoonotic diseases significant to human health, including leishmaniasis and bartonellosis [4].

Control programs for these two disease vectors rely largely upon the use of insecticides. For control of the cattle tick, the USDA implemented CFTEP, which mandates a quarantine zone, dipping of all imported cattle into organophosphate (e.g. coumaphos) solutions, and a 7–14 day quarantine period [4–6]. Similarly, sandfly control is largely based on insecticides through the use of indoor residual spraying with pyrethroids and organophosphates, and the use of insecticide treated bednets is a successful and sustainable method for malaria control that has also been evaluated for control of Phlebotomine sandflies [7–11].

Abbreviations: AChE, Acetylcholinesterase; *Ag*, *Anopheles gambiae*; ATCh, acetylthiocholine iodide; *Bm*, *Rhipicephalus (Boophilus) microplus*; CFTEP, Cattle Fever Tick Eradication Program; CS, catalytic site; *Dm*, *Drosophila melanogaster*; DMSO, dimethyl sulfoxide; DTNB, 5,5'-dithiobis-(2-nitro)benzoic acid; *h*AChE, recombinant human AChE; IC_{50}, inhibitory concentration needed to inhibit 50% of the enzyme activity; OECD, Organization for Economic Co-operation and Development; OP, Organophosphate; *Pp*, *Phlebotomus papatasi*; PS, peripheral site; r*Bm*AChE1, recombinant *Boophilus microplus* acetylcholinesterase, isoform 1; r*Pp*AChE, recombinant *Phlebotomus papatasi* acetylcholinesterase; SR, selectivity ratio; *Tc*, *Torpedo californica*; USDA, United States Department of Agriculture.

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http://dx.doi.org/10.1016/j.pestbp.2013.03.005

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 insecticides, 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 *Ag*AChE and *h*AChE, 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 r*Bm*AChE1 and *Pp*AChE 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 ^{1}H 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.

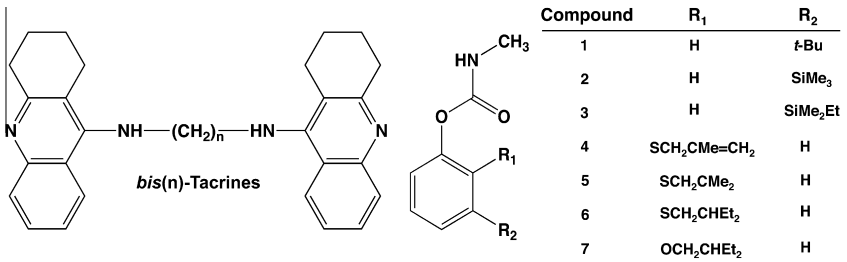
2.2. Molecular homology modeling

Homology model of the *Bm*AChE1 was constructed in ICM [31]. X-ray structure from the Protein Data Bank (PDB ID 1ACJ – the complex of *Torpedo Californica* 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 Bm*AChE1 (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 cDNA of *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 *Bm*AChE1-1203U29X (CTTCTTCTTGCAATACTTCTTCGGATTTC) and *Bm*AChE1-1181L22 (GAACCTTCGTTTGCGTTAGAAC) were utilized (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 mutagenized 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: r*Bm*AChE1 and mutated r*Bm*AChE1 (W384F), prepared as described above, r*Pp*AChE, *h*AChE, bovine brain homogenate, and *Ag*AChE homogenate. *Ag*AChE and bovine brain homogenate enzyme was prepared from groups of ten whole non-blood fed adult female mosquitoes or



Compound	R_1	R_2
1	H	*t*-Bu
2	H	$SiMe_3$
3	H	$SiMe_2Et$
4	$SCH_2CMe{=}CH_2$	H
5	SCH_2CMe_2	H
6	SCH_2CHEt_2	H
7	OCH_2CHEt_2	H

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.