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Insect-specific irreversible inhibitors of acetylcholinesterase in pests including the bed bug, the eastern yellowjacket, German and American cockroaches, and the confused flour beetle

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ABSTRACT

Insecticides directed against acetylcholinesterase (AChE) are facing increased resistance among target species as well as increasing concerns for human toxicity. The result has been a resurgence of disease vectors, insects destructive to agriculture, and residential pests. We previously reported a free cysteine (Cys) residue at the entrance to the AChE active site in some insects but not higher vertebrates. We also reported Cys-targeting methanethiosulfonate molecules (AMTSn), which, under conditions that spared human AChE, caused total irreversible inhibition of aphid AChE, 95% inhibition of AChE from the malaria vector mosquito (*Anopheles gambia*), and >80% inhibition of activity from the yellow fever mosquito (*Aedes aegypti*) and northern house mosquito (*Culex pipiens*). We now find the same compounds inhibit AChE from cockroaches (*Blattella germanica* and *Periplaneta americana*), the flour beetle (*Tribolium confusum*), the multi-colored Asian ladybird beetle (*Harmonia axyridis*), the bed bug (*Cimex lectularius*), and a wasp (*Vespula maculifrons*), with IC_{50} values of ~1–11 μM. Our results support further study of Cys-targeting inhibitors as conceptually novel insecticides that may be free of resistance in a range of insect pests and disease vectors and, compared with current compounds, should demonstrate much lower toxicity to mammals, birds, and fish.

1. Introduction

Wasps, beetles, cockroaches, and bed bugs are insects of medical and economic importance. Some aggressive species of wasps (or *Vespula*) deliver multiple painful stings with venom that can cause allergenic responses and even death from anaphylactic shock [21]. Invasive species of *Vespula* exert serious impact on the biodiversity of native ecosystems [3]. The *Tribolium* beetles, which infest flour and grain stores, contaminate food with carcinogenic quinoles [16,27,31], and also cause allergenic diseases [1]. The ubiquitous cockroaches deposit feces that are a main source of household allergens [18]. Last, obligate blood-sucking bed bugs are regaining their widespread range and formidable reputation as nearly ineradicable pests [13,32]. The resurgence of bed bugs in the United States, Australia and Europe catapulted these insects into prominence as the urban pest problem of the decade. Bed bug infestations are now common in houses and apartments, hotels, hospitals, college dormitories, school rooms, and vehicles [4,9,29,34]. Bed bugs can harbor at least 28 human pathogens. Although disease transmission has not been demonstrated to date, they impact human welfare in many ways, including acute discomfort from bites and feeding, delayed reactions to bug saliva, allergen production, mental stress, and social stigma. Successful eradication of bed bugs is difficult, owing to the small size and cryptic nature of the species, their ease of transport, and their widespread resistance to insecticides. Thus, at present, there is a rising need to treat for bed bugs, cockroaches and other residential pests, yet available agents for such control are either inadequately effective or unsuitable for use close to food or dwellings, due to their human toxicity.

We are exploring a strategy to develop effective insecticides whose risk of mammalian toxicity is greatly reduced by targeting unique features of insect AChE. Many disease-transmitting and agricultural insect pests, including cockroaches and beetles, have two acetylcholinesterase (AChE) genes (AP and AO) [2,7,8,12,14,15,20,30,35]. Sequence analysis and structural genomics studies [22,23] along with site-directed mutagenesis work with lancelet AChE [28] have revealed a free cysteine (Cys) residue at the entrance to the active site of AP-AChE

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in lower organisms but not at that of AO-AChEs and AChEs from mammals, birds and fish. We previously reported that a methanethiosulfonate-containing molecule designed to target this Cys residue can irreversibly inhibit all AChE activity extracted from aphids [25] and >80% of the AChE activity from disease-transmitting mosquitoes [24] without affecting human AChE. Here we provide further evidence that Cys-targeting inhibitors with an advantageous margin of safety might be developed for use against a wide range of insect pests and disease vectors.

2. Materials and methods

2.1. Insects and chemicals

Cockroaches were adult and 5th instar male *Blattella germanica* (L., field collected strains from Cincinnati, New York, and Minneapolis also an Orlando-laboratory susceptible strain), along with adult and 7th instar male *Periplaneta americana* (L.). Beetles were the multi-colored Asian ladybird beetle, *Harmonia axyridis* (Pallas), and the confused flour beetle, *Tribolium confusum* (Jacquelin du Val). Also collected were one wasp, the eastern yellowjacket, *Vespula maculifrons* (Buysson) and a bed bug, *Cimex lectularius* (L.). Buffer constituents, acetylcholine iodide, and miscellaneous laboratory reagents were obtained from Sigma–Aldrich (St Louis, MO, USA). Tritiated acetylcholine (99 mCi/mM) was purchased from New England Nuclear (Waltham, MA, USA). *N*,*N*,*N*-Trimethyl-17-(methylsulfonylthio)heptadecan-1-aminium bromide (AMTS17) and *N*,*N*,*N*-trimethyl-18-(methylsulfonylthio)heptadecan-1-aminium bromide (AMTS18) were synthesized as previously described [25].

2.2. Measurement of AChE inhibition

Insects were rapidly frozen and stored at −80 °C until use. For experiments involving exposure to test compounds and subsequent dialysis, portions of one to six insects were homogenized twice for 10 s each in ground glass homogenizers containing 1 mL of ice-cold 0.09% NaCl, 0.1 M sodium phosphate at pH 7.4 and 0.1% BSA. For *B. germanica* and *V. maculifrons* specimens, sections containing the thorax and head were detached and homogenized. For *P. americana* only the head was assayed. Four to six *T. confusum* beetles were homogenized for assay. An *H. axyridis* specimen was halved and the portion containing the head was homogenized. In all cases the resulting fine suspensions were diluted 30-fold in homogenization buffer before treatments and assays, and they were thoroughly re-suspended as each aliquot was transferred to the reaction tubes. This procedure yielded duplicate agreements within ±2% in assays of insect AChE activity (see below).

After sample exposure to inhibitors (normally 1 h), small molecules were removed before determinations of AChE activity. Most data reported in this article were obtained after dialysis for 16–24 h against two changes of the buffer in more than 100-fold excess. Control experiments with sentinel samples of AChE demonstrated removal of all traces of inhibitory activity by this procedure. More precise time-course data were obtained through rapid inactivation of the test inhibitor by addition of reduced glutathione (final concentration of 500 μM) [21].

The AChE assays were carried out using an established radiometric technique in which product (^{3}H-labeled acetic acid) liberated enzymatically from substrate (^{3}H-labeled acetylcholine, 50 nCi in a final reaction volume of 100 μL at pH 7.4) is partitioned into 4 mL of toluene-isoamyl alcohol (5:1, v/v) with scintillation fluor [5,11]. Routine substrate concentration was 0.1 μM. This condition allowed maximum sensitivity (active samples more than 10 times the buffer-only blanks) with small samples (5 μg wet weight equivalent) and high temporal resolution (assay times as short as 5 min). Also, because of the low substrate concentrations a reversible 50% inhibition was expected to occur at concentrations near the true K_i. When necessary, substrate concentration was adjusted by diluting stock material (99 mCi/mM) with unlabeled acetylcholine chloride. Assay duration, at room temperature, was rigorously controlled to ensure that signal was robust (>5 times blank value) and remained linear with respect to time and amount of sample present (typical conditions, 5 min for concentrated samples or up to 4 h for highly dilute or strongly inhibited samples). Inhibition data were fitted with SigmaPlot (Systat Software, San Jose, CA) to a 4-parameter sigmoidal function: $f = y_0 + a/(1 + \exp(-(x - x_0)/b))$.

3. Results

3.1. Dose response analysis

Insect specimens were homogenized, exposed to AMTS17 (or a close homolog, AMTS18), and then assayed for irreversible AChE inhibition. Dose response analyses were used to calculate IC_{50} values for each species in a paradigm involving 1 h exposure to agent followed by overnight dialysis (Table 1). Of all species assayed in the present study, AChE from the flour beetle, *T. confusum*, was most susceptible to irreversible inhibition by AMTS17 and AMTS18 with IC_{50} values of 1.3 and 1.6 μM, respectively. Concentrations approximately 5-fold higher were required to achieve a given level of irreversible inhibition with AChE from the multi-colored Asian

Table 1
IC_{50} values calculated from dose response analysis of insect homogenates exposed to AMTSn.

	Strain	AMTS17	AMTS18
		IC_{50} (μM)	IC_{50} (μM)
Tribolium confusum Jacquelin du Val (adult mixed sex)	–	1.27	1.55
Harmonia axyridis (Pallas)	–	6.49	–
Cimex lectularius (L.)	–	5.16	–
Cimex lectularius (Wild)	–	5.85	–
Blattella germanica (L.), 5th instar male	Minneapolis	6.11	6.93
Blattella germanica (L.), 5th instar male	Cincinnati	8.15	–
Blattella germanica (L.), 5th instar male	New York	8.86	–
Blattella germanica (L.), 5th instar male	Orlando	7.41	–
Blattella germanica (L.), adult male	Minneapolis	8.16	–
Blattella germanica (L.), adult male	Cincinnati	7.58	–
Blattella germanica (L.), adult male	New York	6.60	–
Blattella germanica (L.), adult male	Orlando	7.20	–
Periplaneta americana (L.), 7th instar male	Scott	10.6	–
Periplaneta americana (L.), adult male	Scott	8.27	–
Vespula maculifrons (Buysson)	–	5.63	–