Actions on mammalian and insect nicotinic acetylcholine receptors of harmonine-containing alkaloid extracts from the Harlequin ladybird *Harmonia axyridis*

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ABSTRACT

The harlequin ladybird, *Harmonia axyridis* (*H. axyridis*), possesses a strong chemical defence that has contributed to its invasive success. Ladybird beetle defensive chemicals, secreted in response to stress and also found on the coating of laid eggs, are rich in alkaloids that are thought to be responsible for this beetle's toxicity to other species. Recent studies have shown that alkaloids from several species of ladybird beetle can target nicotinic acetylcholine receptors (nAChRs) acting as receptor antagonists, hence we have explored the actions of alkaloids of the ladybird *H. axyridis* on both mammalian and insect nAChRs. Electrophysiological studies on native and functionally expressed recombinant nAChRs were used to establish whether an alkaloid extract from *H. axyridis* (HAE) targeted nAChRs and whether any selectivity exists for insect over mammalian receptors of this type. HAE was found to be an inhibitor of all nAChRs tested with the voltage-dependence of inhibition and the effect on ACh EC_{50} differing between nAChR subtypes. Our finding that an HAE fraction consisting almost entirely of harmonine had a strong inhibitory effect points to this alkaloid as a key component of nAChR inhibitory actions. Comparison of HAE inhibition between the mammalian and insect nAChRs investigated indicates some preference for the insect nAChR supporting the view that investigation of ladybird alkaloids shows promise as a method for identifying natural product leads for future insecticide development.

KEYWORDS

Nicotinic acetylcholine receptor; alkaloid; harmonine; antagonist; patch-clamp; voltage-clamp.

ABBREVIATIONS

HAE, Harlequin alkaloid extract.

1. INTRODUCTION

Ladybird beetles (Coleoptera: Coccinellidae) chemically defend themselves by "reflex bleeding" and by coating laid eggs with a defensive fluid that can repel insect predators and competing insect species (Happ and Eisner, 1961). They also cause toxicity when ingested by other insect species (Phoofolo and Obrycki, 1998; Hemptinne *et al.*, 2000; Santi and Maini, 2006) and birds (Marples *et al.*, 1989). Endogenously produced alkaloids are a component of these defensive secretions (Laurent *et al.*, 2002; Haulotte *et al.*, 2012) and are postulated to account for this toxicity. The defensive chemistry of the harlequin ladybird, *Harmonia axyridis,* is of particular interest, as this species has successfully invaded from the Far East and established itself across the Asian and European continents (Roy *et al.*, 2016). When *H. axyridis* was introduced for biological control purposes, unfortunately it outcompeted other ladybird beetle species (Roy and Wajnberg, 2008). Examination of the interactions between *H. axyridis* and competing predator species has shown that *H. axyridis* eggs have a broader range of toxicity to competitors in comparison to eggs of competing ladybird species (Sato and Dixon, 2004; Kajita *et al.*, 2010; Sloggett and Davis, 2010; Katsanis *et al.*, 2013). This has led to multiple investigations into the defensive chemicals of *H. axyridis*. Of particular interest has been the alkaloid harmonine, with its broad antimicrobial activity (Alam *et al.*, 2002; Rohrich *et al.*, 2012), toxicity to parasitic trematodes of the genus *Schistosoma* and inhibition of the enzyme acetylcholinesterase (Kellershohn *et al.*, 2019).

Ladybird beetles themselves have been deployed as a home remedy to soothe toothache (Majerus, 2016) and ion channels including nAChRs are analgesic targets (Umana *et al.*, 2013) as well as being targets for alkaloids (Wink, 2000), leading to the hypothesis that ladybird alkaloids could target ion channels. This was confirmed when investigation of several azaphenalene alkaloids from multiple ladybird beetle species

were found to inhibit mammalian nicotinic acetylcholine receptors (nAChRs) (Leong *et al.*, 2015). The nAChRs are members of the Cys-loop family of ligand-gated ion channels (Cys-loop LGICs) (Thompson *et al.*, 2010). The neurotransmitter roles of these pentameric, transmembrane molecules are well documented and individual receptors consist of 5 highly homologous subunits, further sub-divided into α and non-α types. The α subunits possess a pair of adjacent cysteines in loop C, one of the 6 loops (A-F) that make up the ACh binding site located at the interface between adjacent subunits (Changeux and Edelstein, 2006). Typically loops A, B and C are provided by the α subunit and loops D, E and F are donated by the non-α subunit. 9 α, 4 β, 1 γ, 1 δ and 1 ε subunits are found in humans with specific combinations of subunits occurring in particular areas within the nervous system (Wooltorton *et al.*, 2003) and at neuromuscular junctions (Kalamida *et al.*, 2007).

Insect nAChRs play important roles in behaviour as ACh is the primary afferent neurotransmitter in these organisms (Breer and Sattelle, 1987) and they are also targets for insecticides (Gepner *et al.*, 1978; Sattelle, 1980; Ihara *et al.*, 2018). Cartap (Lee *et al.*, 2003), the commercial derivative of the natural annelid toxin, Nereistoxin (Sattelle *et al.*, 1985), Spinosyns (Sparks *et al.*, 2001) and neonicotinoids, which show similarities to nicotine and epibatidine (Matsuda *et al.*, 2009), have all been successfully developed as commercial insecticides, with imidacloprid being the first commercially successful compound (Bai *et al.*, 1991; Kagabu, 2011). Since then acetamiprid, clothiandin, nitenpyram, dinotefuran, thiacloprid and thiamethoxam have been developed (Ihara *et al.*, 2018). Flupyradifurone is a butenolide insecticide that activates insect nAChRs (Nauen *et al.*, 2015). The sulfoximine, sulfoxaflor, is also an agonist of insect nAChRs (Oliveira *et al.*, 2011; Watson *et al.*, 2011). Triflumezopyrim is a recent

addition to insecticides targeting insect nAChRs acting as an antagonist at the ACh binding site (Cordova *et al.*, 2016).

Other commercially successful groups of insecticides target other insect ion channels including GABA receptors (Buckingham *et al.*, 2017), ryanodine receptors (Cordova *et al.*, 2007) and sodium channels (Soderlund, 2010). However, the actions of all insecticides will ultimately suffer from the development of resistant pest insect populations resulting in a need for a pipeline of alternative insecticides (Soderlund, 2008; Sparks *et al.*, 2012; Bass *et al.*, 2015; Troczka *et al.*, 2015; Buckingham *et al.*, 2017) and improved pest management strategies (Sparks and Nauen, 2015). Neonicotinoids are now facing intense scrutiny due to their adverse actions on pollinators (Stanley *et al.*, 2015) and insectivorous birds (Hallmann *et al.*, 2014) hastening the need for alternatives. Investigation of natural products has proved to be a viable pathway for the development of novel insecticide leads (Thompson *et al.*, 2000). As ladybird alkaloids target mammalian nAChRs, there exists the possibility that insect nAChRs are also targeted due to their toxic actions on other insect species.

Harmonine-containing alkaloid extracts from *H. axyridis* were therefore examined for their activity on both insect and mammalian nAChRs. Detection of any target-site selectivity for insects over mammalian nAChRs would make *H. axyridis* alkaloids worthwhile leads to pursue further in the search for novel insect control chemicals.

2. MATERIALS & METHODS

2.1 Chemical reagents and nucleic acids

ACh was obtained from Sigma-Aldrich. cDNA clones of rat neuronal nAChR subunits (α3, α4, β2, β4) were acquired from the laboratory of Professor Stephen

Heinemann at the Salk Institute for Biological Studies. The cDNA clone for the human α7 and the chick β2 nAChR subunits were a gift from Professor Marc Ballivet. The *Drosophila* nAChR Dα2 was provided by Professor Heinrich Betz. Plasmids were linearised and cRNA transcribed using the mMessage mMachine transcription kit (Ambion).

2.2 Whole alkaloid extraction

Adult *H. axyridis* beetles were collected from the grounds of University Park (Nottingham) and Rothamstead Research (Harpenden, Hertfordshire), and stored at -20 °C until extraction. A whole alkaloid extract of *H. axyridis* (HAE) was obtained by initially freezing the beetles (ca. 5000 weighing ca. 187 g) in liquid nitrogen, crushing with a pestle and mortar and preparing a whole organism extract by soaking the crushed material in methanol (2 x 250 mL) for 24 h at room temperature. The methanolic extracts were filtered using filter paper (Grade 4, Whatman), combined and dried using a rotary evaporator (BUCHI, Switzerland). The residue (ca. 8 g) was then subjected to acid-base extraction at room temperature using 1 M HCl (50 mL) and diethyl ether (3 x 20 mL) to remove lipoidal material, followed by adjustment to ca. pH 10 – 12 using 2 M NaOH and extraction with dichloromethane (3 x 20 mL). The combined organic layers were washed with saturated NaCl solution, dried using anhydrous $MgSO_4$ and evaporated *in vacuo* to yield a residue (ca. 450 mg). The residue was re-dissolved in dichloromethane and aliquots of residue (300 μg and 500 μg) were measured for storage in glass ampoules sealed under nitrogen. The presence of alkaloids in HAE was confirmed using Dragendorff's reagent.

Crude fractions of HAE were prepared by using 5 mg HAE dissolved in methanol. HAE was loaded onto silica glass plates (20 cm^3, Fluka) and plates were run using methanol with 1 % ammonium hydroxide as the eluting solvent. Two fractions were

clearly visible without staining, and Dragendorff's reagent confirmed that these fractions were discrete, one mobile and one that remained at the loading level. The silica containing each of the two fractions was collected into glass vials and extracted in methanol. The supernatant was removed to clean glass vials and dried under nitrogen to yield two dried extracts: the mobile fraction A (560 µg) and the stationary fraction B (1.27 mg).

Coupled Gas Chromatography-Mass-Spectrometry (GC-MS) analysis of extracts was performed using a Waters Autospec Ultima mass spectrometer coupled to an Agilent 6890 GC fitted with a HP-1 capillary column (50 m x 0.32 mm id, 0.52 µm film thickness) and a cool on-column injector. Ionization was by electron impact (70 eV, source temperature 220 °C). Helium was the carrier gas. The oven temperature was maintained at 30 °C for 5 min, and then programmed at 5 °C/min to 250 °C. Identification was confirmed by comparison of spectral data with published values (Abel et al., 2015).

2.3 TE671 cell and locust neuron culture

TE671 cells express embryonic human muscle-type nAChRs (composed of $(\alpha1)_2\beta1\delta\gamma$ subunits containing the γ embryonic subunit rather than the adult ε subunit) (Luther *et al.*, 1989). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and supplemented with 10 % fetal calf serum, 2 mM glutamine, 10 IU/mL penicillin and 10 µg/mL streptomycin (Sigma-Aldrich), and incubated at 36.5 °C in a 5 % CO_2 atmosphere. Cells were grown in 25 cm^2 flasks and divided 1:10 when they were approximately 75 % confluent. For whole-cell patch-clamp electrophysiological recordings, dividing cells were plated onto pieces of glass coverslip (5x20 mm) in 35-mm Petri dishes containing 2 mL DMEM.

Desert locusts (*Schistocerca gregaria*) were purchased from Livefoods UK Ltd and kept in locust breeding cages maintained at an ambient temperature of 26-28 °C with a 40 W incandescent bulb used to provide a basking spot of 38 °C and a 12:12 h light cycle. Locusts were selected at the 6th instar, cold anaesthetised at 4 °C for 10 min, dipped in 70 % ethanol and decapitated. Locust heads were transferred to cooled Ca^{2+}/Mg^{2+} free Rinaldini's saline (135 mM NaCl, 25 mM KCl, 0.4 mM $NaHCO_3$, 0.5 mM glucose, 5 mM HEPES, pH 7.2 with NaOH). The brain was excised and placed in cooled Ca^{2+}/Mg^{2+} free Rinaldini's saline. The mushroom bodies were dissected and placed in 200 µL of Rinaldini's saline containing 2 mg/mL collagenase (type 1A, Sigma-Aldrich) and 0.5 mg/mL dispase (Boehringer Mannheim UK Ltd). After 15 min incubation at 36.5 °C, the tube was centrifuged at 800 x g for 1 min at room temperature. The supernatant was removed and replaced with 200 µL locust culture medium (5:4 DMEM [supplemented with 10 % FCS, 2 mM Glutamine]:Schneider's insect medium, with 10 IU/mL penicillin and 20 µg/mL streptomycin). The mushroom bodies were then gently triturated through a 200 µL Gilson pipette tip before being distributed over heat-sterilised glass coverslips (5x20 mm) coated in 0.01 % poly-L-lysine (Sigma-Aldrich) that had been placed into 35 mL Petri dishes containing 2 mL locust culture medium. Dishes were incubated at 36.5 °C in a 5 % CO_2 atmosphere and used within 24 h.

2.4 Whole-cell patch-clamp electrophysiology

Whole-cell patch-clamp electrophysiology was carried out on TE671 cells and locust neurons using an Axopatch 200A (Axon Instruments, US) patch-clamp amplifier and recorded to the disk of a PC using WinWCP V4.5.7 software provided by Dr John Dempster, Institute of Pharmacy & Biomedical Sciences, University of Strathclyde, UK. Patch-pipettes were created using borosilicate glass capillaries (World Precision Instruments, US) using a programmable micropipette puller (P-97, Sutter Instruments

Co., US) and filled with a caesium chloride based pipette solution (140 mM CsCl, 1 mM $MgCl_2$, 11 mM EGTA and 5 mM HEPES, pH 7.2) giving resistances of 5-7 MΩ. Solutions were perfused using a DAD-12 Superfusion system (Adams & List Associates, US) fitted with a 100 µm polyamide coated quartz output tube with a solution exchange time of 30 - 50 ms and controlled by WinWCP. The perfusion system was pressurised with compressed nitrogen and solutions applied at 200 mm/Hg. TE671 cells prepared for patch-clamp studies were placed in a perfusion chamber and constantly perfused at a flow rate of 5 mL/min with mammalian Ringer (135 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 10 mM D-glucose, pH 7.4 with NaOH). Locust neuronal cultures were perfused with locust saline (180 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.2 with NaOH).

2.5 *Xenopus laevis* oocyte preparation and cRNA injection

Xenopus laevis oocytes were acquired from the European Xenopus Resource Centre (University of Portsmouth, UK). On arrival, excised ovaries were treated with 0.5 mg/mL collagenase (type 1A, Sigma) in Ca^{2+}-free modified Barth's saline (MBS) (96 mM NaCl, 2 mM KCl, 5 mM HEPES, 2.5 mM pyruvic acid & 0.5 mM theophylline, pH 7.5) for 1 h at 18 °C to remove the follicular tissue surrounding the oocytes. After washing with Ca^{2+}-free MBS the isolated oocytes were incubated at 18 °C in MBS supplemented with the antibiotic gentamicin (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, 2.5 mM pyruvic acid & 0.5 mM theophylline, pH 7.5 followed by the addition of 0.05 mg/mL gentamicin). Healthy stage IV-V oocytes were selected and 50 nL cRNA was injected using a Nanoliter injector (World Precision Instruments, Inc.). Human α7 was injected at a concentration of 100 ng/µL, rat α3/β4 and rat α4/β2 were injected in a 1:1 ratio at a concentration of 200 ng/µL each and *Drosophila* α2/chick β2 was injected in a 1:1

ratio at a concentration of 1 μg/μL each. Oocytes were incubated for 3-4 days at 18 °C prior to electrophysiological recordings.

2.6 Two-electrode, voltage-clamp electrophysiology

Electrophysiological recordings were obtained from nAChR-expressing oocytes by two-electrode voltage clamp using an Axoclamp 2A voltage clamp amplifier (Axon instruments, USA). An oocyte was transferred to the perfusion chamber using a plastic Pasteur pipette and the bath was perfused (~5 mL/min) with standard oocyte saline (SOS) (100mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES, pH 7.6). Microelectrodes were pulled from borosilicate glass capillaries (GC150TF-4, Harvard Apparatus) using a programmable micropipette puller (P-97, Sutter Instruments Co., US), and they had resistances between 0.5 and 2.5 MΩ when filled with 3 M KCl. The oocyte was voltage-clamped at a holding potential (V_h) of -75 mV. ACh was consistently used as the agonist, and it was applied without or together with HAE extracts via a MPS-2 multi-channel gravity fed perfusion system (World Precision Instruments Inc.). Atropine (0.5 μM) was added to the SOS to prevent any endogenous muscarinic ACh receptor response (Dascal and Landau, 1980). Output currents were transferred using a PCI-6221 A/D converter (National Instrument Corp., US) to a PC and WinEDR software (Dr John Dempster, Institute of Pharmacy & Biomedical Sciences, University of Strathclyde, UK) was used for recording and analysis.

2.7 Data Analysis

WinWCP software was used to measure the peak current and current 1 s after onset of response for patch clamp electrophysiology recordings. WinEDR software was used to measure the peak current and current 15 s after onset of response ("late" current) for two-electrode voltage clamp electrophysiology recordings. Data were normalised as a % of an ACh control response or % of maximal ACh response. Each

plotted data point is the mean ±SEM of recordings from 7-14 cells or 5-8 oocytes. GraphPad Prism 8 was used for all data analysis, graph plotting, curve fitting and statistical tests as well as application of the Woodhull equation. Concentration-inhibition and concentration-response curves were used to calculate IC_{50}s for HAE/HAE fractions and EC_{50}s for ACh using the following equations:

$$\%\ control\ response = 100/(1 + 10^{(LogIC_{50} - X) * Hillslope})$$

or

$$\%\ max\ ACh\ response = max/(1 + 10^{(LogEC_{50} - X) * Hillslope})$$

where X is the concentration of ACh or HAE.

3. RESULTS

3.1 Actions of harmonine-containing alkaloid extracts on human muscle nAChRs and insect neuronal nAChRs

Extraction of alkaloids from adult *H. axyridis* beetles resulted in an alkaloid extract containing multiple compounds (Fig. 1C) including the alkaloid harmonine (Fig. 1D). Further fractioning of the extract resulted in 2 alkaloid fractions, one mobile (fraction A) and one that remained at the loading level (fraction B) (Fig. S1A & B). 5 mg was used for this process of which 560 µg was recovered as fraction A, 1.27 mg was recovered as fraction B and the remaining 3.17 mg was unaccounted for and may have been lost in the re-extraction procedure. Gas chromatography-Mass Spectrometry (GC-MS) analysis revealed fraction B to be harmonine and fraction A to be a mixture of unidentified alkaloids and a low concentration of harmonine (Fig. S1C & D).

The effect of both fractions on human muscle-type nAChRs expressed in TE671 cells and insect neuronal-type nAChRs in locust neurons was investigated using patch-clamp electrophysiology. No inward currents were detected upon application of the

fractions A or B to TE671 cells and locust neurons (Fig. S2). Having established that there were no nicotinic stimulatory effects by the alkaloids, we co-applied the fractions with ACh. Inhibition of the ACh response was observed upon co-application of either fraction A or fraction B with ACh to both TE671 cells and locust neurons (Fig. 2A - D) and concentration-inhibition plots were created in order to obtain IC_{50} values for each fraction (Fig. 3A - D, Table 1).

The peak current amplitude and the current amplitude after 1 s (1 s current) were both measured because some antagonists, notably Philanthotoxin-343, can have a much larger effect on the late current than the peak current (Brier *et al.*, 2003). A greater effect on the late current would imply that HAE inhibition was dependent on the activation of the receptor. Comparison of IC_{50} values of the HAE fractions showed that the 1 s current IC_{50} was lower than the peak current IC_{50} in each case. For fraction A the 1 s IC_{50} was 156-fold lower than the peak IC_{50} for TE671 cells and 28-fold lower for locust neurons. For fraction B the 1 s IC_{50} was 17-fold lower than the peak IC_{50} for TE671 cells and 74-fold lower for locust neurons. This implies that the inhibition was activation-dependent. In addition to the comparison of the effect of the HAE fractions on the peak and 1 s current, a comparison of the HAE fractions between TE671 cells and locust neurons was made to determine whether selectivity exists for insect neuronal-type nAChRs over mammalian muscle-type nAChRs (Fig. 3E & F). One-way ANOVA revealed that in all comparisons, fraction B was significantly more potent on locust neuron nAChRs than on the TE671 cell nAChR (peak $P \leq 0.05$ and 1 s $P \leq 0.0001$). Fraction B was also significantly more potent than fraction A in its action on nAChRs from both cell types (peak $P \leq 0.05$ and 1 s $P \leq 0.0001$).

Table 1. IC_{50} values for inhibition by HAE fractions A and B of the peak and 1 s current of locust neuronal nAChRs and human muscle-type nAChRs expressed TE671 cells.

Cell/nAChR type	Current	IC_{50}, µg/mL (95 % CI)	
		Fraction A	Fraction B
TE671 (human muscle-type)	Peak	745 (57.4 – 9669)	69.6 (24.9 – 194)
	1 s	4.77 (2.12 – 10.7)	1.68 (1.16 – 2.45)
Locust (insect neuronal-type)	Peak	90.2 (25.1 – 325)	5.30 (2.87 – 9.88)
	1 s	3.23 (1.69 – 6.18)	0.072 (0.038 – 0.140)

3.2 Mode of action studies on human muscle-type nAChRs

As there was a limited yield of fractions from the thin-layer chromatography process, the mode of action of whole HAE rather than the separate fractions was investigated. Furthermore, the availability of whole HAE was also limited so mode of action studies were conducted on TE671 cells only. The effect of HAE on the ACh EC_{50} was explored in order to determine whether there was competition between HAE and ACh at the agonist binding site of the nAChR. Concentration-response plots in the presence and absence of HAE were constructed (Fig. 4A & B) with a HAE concentration of 30 µg/mL. Comparison of the plateau of the curves showed a lower plateau (80 % of ACh control for peak and 41 % of ACh control for 1 s) in the presence of HAE. EC_{50} values of ACh control vs in the presence of HAE were 3.30 µM (95 % CI = 2.61 – 4.29) vs 6.31 µM (95 % CI = 3.61 – 11) for peak current and 2.26 µM (95 % CI = 1.6 – 3.19) vs 1.48 µM (95 % CI = 0.07 – 31.2) for 1 s current. Observation of lowered plateau values indicate a non-competitive mode of action for HAE on mammalian muscle-type nAChRs in TE671 cells, however, a significant difference was observed (P = 0.0433, extra sum-of-squares F-test, GraphPad Prism 8) for the increase in peak current EC_{50} indicating a minor component of competitive action.

The effect of changes in V_h on the inhibitory activity of HAE was also investigated in order to determine potential binding of HAE to the channel pore (Fig. 4C & D). Concentration-inhibition plots were constructed over the HAE concentration range of 0.1 – 100 μg/mL. HAE was co-applied with 10 μM ACh at V_h of +50 mV, -50 mV, -75 mV, -100 mV and -120 mV (Fig. 4C). The inhibition of the peak current was low (15 – 20 % inhibition with 100 μg/mL HAE) such that accurate curves could not be fitted nor IC_{50} values calculated. The 1 s current IC_{50} values were reduced at more negative V_h values; IC_{50} values were plotted against holding potential (Fig. 4D) and a regression fitted with the Woodhull equation (Woodhull, 1973). The mixed composition of HAE meant that an accurate Z value could not be assigned, so Z and δ were combined. The slope of the Woodhull-fitted regression differed significantly from zero ($P = 0.012$) indicating strong voltage-dependence of inhibition by HAE on muscle-type nAChRs in TE671 cells. However, there was still substantial inhibition at +50 mV suggesting that either binding was not deep in the pore, or an additional binding site outside of the pore is present.

3.3 Exploring selectivity by comparing actions on receptor subtypes with different subunit composition

In order to further investigate selectivity between mammalian and insect nAChRs, a comparison of the effects of HAE on mammalian neuronal-type nAChRs was needed as the comparison between TE671 cells and locust neurons is a comparison between mammalian muscle-type and insect neuronal-type nAChRs. The *Xenopus* oocyte heterologous expression system and two electrode voltage-clamp electrophysiology technique was used to investigate the effects of HAE on mammalian neuronal-type human α7, rat α4β2, rat α3β4 and the hybrid *Drosophila* α2/chick β2

(Dα2/β2) nAChRs clamped at V_h = -75 mV. Inhibition by HAE of each receptor was observed (Fig. 5A - D) and concentration-inhibition plots were constructed in order to obtain and compare IC_{50} values (Fig. 6A - F, Table 2). For a more direct comparison with the 1 s current measurement from the TE671 cell and locust neuron studies, the current was measured 15 s from response onset (late current). This could only be achieved with the more slowly desensitising rat α4β2 and rat α3β4 nAChRs and late current IC_{50} values are given in Table 2. When taking only peak current into account, the Dα2/β2 IC_{50} was 3.34 fold lower than the lowest mammalian nAChR IC_{50} indicating some selectivity for Dα2/β2. Statistical comparison of the IC_{50} values (Fig. 6C) shows the Dα2/β2 peak current IC_{50} to be significantly lower than all mammalian receptors investigated ($P < 0.0001$ for Dα2/β2 vs α4β2, $P = 0.0159$ for Dα2/β2 vs α7, $P = 0.0065$ for Dα2/β2 vs α3β4, extra sum-of-squares F-test, Graphpad Prism 8). However, there is a substantial effect on the late current of α3β4 with a reduction to 17 % of the ACh control response upon application of the lowest concentration of HAE (0.003 μg/mL).

Table 2. IC_{50} values for inhibition by HAE of human α7, rat α4β2, rat α3β4 and the hybrid *Drosophila* α2/chick β2 (Dα2/β2) nAChRs expressed in *Xenopus* oocytes (* 95 % CI could not be calculated; N/A = no late current).

Receptor	IC_{50}, μg/mL (95 % CI)	
	Peak Current	Late Current
α7	2.30 (1.67 – 3.17)	N/A
α4β2	3.24 (1.47 – 8.65)	0.431 (0.233 – 0.804)
α3β4	1.47 (0.708 – 2.88)	<<0.03 (*)
Dα2/β2	0.440 (0.211 – 0.910)	N/A

3.4 Further exploration of mode of action studies on receptor subtypes with different subunit compositions

The increased solution volumes required for investigation using the rapidly perfused *Xenopus* oocyte expression system was demanding on HAE availability. Hence multiple concentration-inhibition curves could not be constructed to investigate voltage-dependence of HAE inhibition. As an alternative approach, a voltage ramp from -90 mV to +50 mV over a period of 1 s was applied during the steady-state phase of the rat α4β2 and rat α3β4 nAChR currents. 0.3 μg/mL HAE co-applied with 100 μM ACh was used for α3β4 and 3 μg/mL HAE co-applied with 10 μM ACh was used for α4β2. Measurable inward currents were observed when the V_h reached approximately -40 mV for both receptors and so the current in the range -40 mV to -90 mV was normalised to the control ACh response (Fig. 7A). The level of HAE inhibition for α4β2 significantly changed from approximately 75 % of the control response at -40 mV to 44 % at -90 mV (comparison of the -50 mV to -40 mV and -90 mV to -80 mV ranges, P = 0.0006, paired t-test) indicating voltage-dependence of HAE inhibition on α4β2. The level of HAE inhibition for α3β4 remained consistent at approximately 25 % of the control ACh response for the entire V_h range indicating HAE inhibition is not voltage-dependent on α3β4. As human α7 is a rapidly desensitising receptor a voltage ramp could not be applied. An alternative method of applying an ACh control (100 μM ACh) and manually changing the V_h in 25 mV steps from -25 mV to -125 mV followed by co-application of ACh and HAE (1 μg/mL) at the same V_hs was used (Fig. 7B). One-way ANOVA revealed no significant differences in the percentage of inhibition across the V_h range tested.

The effect of HAE on the ACh EC_{50} was also examined with α3β4 and α4β2 (Fig. 8A & B). HAE concentrations of 0.3 μg/mL and 3 μg/mL were used for α3β4 and α4β2 respectively in order to produce a more measurable level of inhibition. EC_{50} values of ACh control vs in the presence of HAE were 161 μM (95 % CI = 138 – 187) vs 184 μM (95 % CI = 98.8 – 254) for α3β4 and 19.9 μM (95 % CI = 9.66 – 38.4) vs 241 μM (95 % CI

= 100 – 386) for α4β2. The maximum response was reduced to 73.4 % of the control response for α3β4 with no effect on the maximum response of α4β2. Only the increase in EC_{50} value observed for α4β2 was found to be significant ($P < 0.0001$, extra sum-of-squares F-test, GraphPad Prism 8) indicating competition between HAE and ACh at the agonist binding site of α4β2. The observed reduction in maximum response with minimal (NS) shift in the ACh EC_{50} for α3β4 suggest non-competitive inhibition by HAE.

4. DISCUSSION

HAE was shown to be an inhibitor of the nAChR response of human α7, rat α3β4, rat α4β2 and the hybrid Dα2/β2 nAChRs expressed in *Xenopus* oocytes as well as the nAChR responses of TE671 cells and locust neurons (Fig. 2 & 5). Examination of the inhibition of the observed peak current showed selectivity for a native insect and an insect/vertebrate hybrid nAChR in each comparison indicating potential selectivity of HAE for insect over mammalian nAChRs (Fig. 3 & 6). The inhibition of the responses of TE671 cells and locust neurons showed a greater inhibitory effect on the current 1 s after application and so this was examined in the responses of the slowly desensitising nAChRs expressed in *Xenopus* oocytes by examining the current 15 s after application (late current) (Fig. 6B). This revealed a larger effect on the late current consistent with the results observed in TE671 cells and locust neurons. The inhibition of the late current of rat α3β4 was so great that concentrations lower than the range tested in these studies will need to be applied in order to determine an appropriate IC_{50}. A greater inhibitory effect after the onset of response indicates HAE inhibition is at least partly activation-dependent.

Further studies were carried out on mammalian nAChRs in order to explore the mode of action of HAE inhibition. Inhibition was shown to be voltage-dependent in

TE671 cells indicating binding to the pore region of the open channel (Brier *et al.*, 2003) and analysis of voltage-dependency using the Woodhull equation (Woodhull, 1973) was consistent with actions as an open channel blocker. In addition to this, HAE still showed some inhibition at the positive V_h of +50 mV (Fig. 4C), albeit at lower levels than observed at negative V_hs, indicating that the binding site is either to a site not deep in the pore, or to an additional site outside of the pore. Voltage-dependent HAE inhibition was found to be inconsistent across all mammalian nAChRs investigated. For example, the inhibition of human α7 and rat α3β4 was found to be voltage-independent while inhibition of rat α4β2 was found to be voltage-dependent (Fig. 7). Differences in the voltage-dependency of inhibition indicate binding to the pore region only in muscle-type nAChRs and α4β2 neuronal-type nAChRs with a distinct type of binding site being present on α7 and α3β4 nAChRs. This is consistent with the observation of inhibition at +50 mV pointing to a potential binding site outside of the pore.

The possibility of binding to the ACh binding site was investigated by analysing the effect of the presence of HAE on the EC_{50} of ACh for each of the nAChRs. In TE671 cells (Fig. 4A & B) the upper plateau of the EC_{50} plot was reduced by HAE rather than a large increase in the EC_{50}, which reveals no competition between HAE and ACh for the ACh binding site. This was not the case for all nAChRs tested as the same study revealed a reduction of the upper plateau for α3β4 indicating no competition for the ACh binding site, however, a lack of reduced upper plateau and a significant increase in the EC_{50} for α4β2 did indicate competition for the ACh binding site for this nAChR subtype (Fig. 8). These investigations point to potential binding of HAE at both the pore and the ACh binding site of the nAChRs investigated, with the relative extent of each being dependent on receptor subtype. As HAE is a mixture of compounds, further separation needs to be carried out. This will assist in determining whether our findings are

attributable to one compound (e.g. harmonine) binding to different receptor regions, depending on nAChR subtype. Alternatively, our findings could be explained in terms of the binding of different HAE components to distinct binding sites.

Separating the components of HAE proved challenging for a number of reasons. Although it would have been desirable to isolate, identify and quantify all of the individual alkaloid compounds from the mixture, this was not possible. When subjected to reverse phase HPLC, it was possible to separate compounds, but harmonine, the major alkaloid present, could not be visualised with UV absorbance. We considered deploying HPLC coupled with secondary mass spectrometry, but the low-throughput nature of the technique precluded this approach. Liquid chromatography was attempted to increase the quantity of alkaloid extract that could be separated and fractions collected. Multiple attempts with different stationary phases, solvents and solvent gradients failed to separate alkaloids effectively. Preparative scale TLC enabled separation of harmonine (fraction B) from the other alkaloid components (fraction A) within the alkaloid extract (Fig. S1A & B); this was confirmed with mass spectrometry (Fig. S1C & D). From 5 mg of whole alkaloid extract, 1.27 mg of harmonine and 560 µg of unidentified alkaloids with a very low concentration of harmonine were recovered. This confirmed that harmonine is the predominant alkaloid within HAE and because fraction B showed a greater inhibitory effect (Fig. 3, Table 1), harmonine is the prime candidate alkaloid responsible for nAChR inhibition. An alternative method of obtaining harmonine to the isolation carried out in this study is to synthesise harmonine *de novo*. Initially 2 syntheses were published consisting of 19 and 15 steps (Braconnier *et al.*, 1985; Enders and Bartzen, 1991) but more recently multiple separate shorter syntheses consisting of 5 - 6 steps have been published (Philkhana *et al.*, 2014; Abel *et al.*, 2015; Nagel *et al.*, 2015). Employment of these recent syntheses could prove a more reliable

and successful route of obtaining harmonine and permit the production of structural analogues that will address the issues of correct physicochemical properties as well as compound stability.

The results of this study along with the results of the investigation of several azaphenalene alkaloids from other species of ladybird beetle show that antagonist actions on nAChRs are characteristic of ladybird beetle alkaloids. The azaphenalene alkaloids displaced binding to the *Torpedo* nAChR of tritiated piperidyl-*N*-(1(2-thienyl)cyclohexyl)-3,4-piperidine ([^{3}H]-TCP), which binds deep within the channel pore, but did not displace binding of [^{3}H]-cytisine which binds to the ACh binding site indicating non-competitive inhibition (Leong *et al.*, 2015). The effects of the azaphenalene alkaloids, precoccinelline and coccinelline, were also examined on muscle-type nAChRs expressed in TE671 cells and pre-coccinelline on human α7 nAChRs expressed in *Xenopus* oocytes. Non-competitive inhibition was observed in each case (Leong *et al.*, 2015). This resembles our own observations on the binding of HAE to the pore region in TE671 cells, however, this binding may be to a shallower region of the pore than the [^{3}H]-TCP binding site. Both nereistoxin and the insecticide derived from it, cartap, are also nAChR inhibitors that displace [^{3}H]-TCP in honeybee (*Apis melifaria*) head membranes (Lee *et al.*, 2003), so HAE could have a different mechanism of inhibition to nereistoxin and cartap.

There exist over 4,200 species of ladybird beetle (Iperti, 1999) and alkaloids with a variety of different structures have been identified in a relatively small number of these species (King and Meinwald, 1996), therefore, investigation into these alkaloids could also yield many more chemicals of interest. Alkaloids are also present in many other arthropod species (Roberts and Wink, 2013) that could also yield new insecticide candidates. A number of arthropod alkaloids have shown activity on both mammalian

and insect ionotropic receptors such as Philanthotoxin from the Egyptian digger wasp *Philanthus triangulum* (Rozental *et al.*, 1989; Brier *et al.*, 2003; Mellor *et al.*, 2003; Kachel *et al.*, 2016). Alkaloids from the venom of the fire ant *Solenopsis saevissma* and *Solenopsis richteri* show activity on the insect nAChR but do not show insecticidal activity in whole insect toxicity tests (David *et al.*, 1984). Harmonine did not cause lethal toxicity when injected into the 7-spot ladybird, *Coccinella septempunctata* (Vilcinskas *et al.*, 2013), and it will be important to pursue such tests on pest species for this and other arthropod alkaloids.

Further investigation of HAE and harmonine after separation will allow a more detailed understanding of the binding region and mode of action. Further examination of their selectivity will be beneficial, as expression of other mammalian and insect neuronal-type nAChRs in *Xenopus* oocytes is possible. The *Drosophila* nAChR subunits Dα1 (Ihara et al., 2014) and Dα5/Dα7 (Lansdell et al., 2012) can also be expressed in *Xenopus* oocytes. Expression of nAChR subunits from other invertebrate species such as the desert locust *Schistocerca gregaria* (Marshall *et al.*, 1990; Amar *et al.*, 1995), the brown dog tick *Rhipicephalus sanguineus* (Lees *et al.*, 2014) and the nematode worm *Caenorhabditis elegans* (Ballivet *et al.*, 1996; Boulin *et al.*, 2008) is also possible so in future it will be of interest to explore whether HAE is more or less selective for other insect/invertebrate neuronal-type nAChRs. As HAE appears to show a degree of selectivity for the insect nAChR, ladybird alkaloids may be of interest as potential sources for lead candidates in the search for novel insecticides.

FIGURE LEGENDS

Figure 1. Ladybird beetle alkaloid extraction overview. Adult *H. axyridis* beetles (A) were collected and used as the source of alkaloids for this study. The alkaloid extraction process (B) involved using methanol to extract the haemolymph (blood analogue in insects) then separating the alkaloids using an acid-base separation followed by thin-layer chromatography to generate fractions. Gas-chromatography – Mass Spectrometry analysis produced a chromatogram (C) showing multiple compounds present in HAE including the alkaloid harmonine (D) known to be present in *H. axyridis*.

Figure 2. **HAE fractions A and B inhibit the ACh response of TE671 cells and locust neurons.** Representative traces showing the inhibition of ACh induced whole-cell currents in both TE671 cells (A & C) and locust neurons (B & D) by fractions of HAE generated by thin-layer chromatography. Both fractions antagonised nAChR currents in both cells types, but fraction B was significantly more active against locust receptors than fraction A and showed selectivity for locust over TE671 nAChR (V_h = -75 mV).

Figure 3. **HAE fractions A and B have a larger inhibitory effect on the late current, with fraction B more effective than fraction A and showing greater selectivity for locust neuron nAChRs.** Increasing concentrations of HAE fractions A and B were individually co-applied with ACh to both TE671 cells (blue) and locust neurons (red) which had been clamped at V_h = -75 mV (A, B, C & D). The peak and 1 s currents were measured and normalised against a control response. A 4 parameter logistic regression curve was fitted to the points, from which IC_{50} values were derived (n = 10 for Locust Fr A, 9 for Locust Fr B, 9 for TE671 Fr A and 12 for TE671 Fr B). The IC_{50} and selectivity values for Fractions A and B were then compared in column charts (E & F) against TE671 (blue) and locust neurons (red) nAChRs. Asterisks (* $P < 0.05$, **** $P < 0.0001$) represent the result of one-way ANOVA comparison of IC_{50} values. Fraction B is significantly more potent to locust nAChR than it is to TE671 and more potent than Fraction A on both TE671 and locust neurons (V_h = -75 mV; error bars show 95 % CI).

Figure 4. **HAE shows non-competitive, voltage-dependent inhibition of human muscle-type nAChRs in TE671 cells.** Concentration-response plots (A & B) showing the current response of TE671 cells to ACh (black) and to ACh co-applied with 30 µg/mL HAE (red). Data were normalised to the peak response to ACh alone. The results show that co-application of HAE with ACh causes the response to plateau at 80 % for peak current and 41 % for 1 s current compared to ACh alone. Rather than a shift to the right, as expected if ACh displaced alkaloid binding, curves instead reflect a non-competitive inhibition of the ACh response. The EC_{50} values were (control and in the presence of alkaloid respectively) 3.35 µM and 6.31 µM for peak and 2.26 µM and 1.48 µM for 1 s currents. Error bars show ±SEM, V_h = -75 mV and n = 12. (C) Concentration-inhibition plot showing the voltage-dependence of HAE inhibition of human muscle-type nAChR. TE671 cells were clamped at holding potentials ranging from +50 to -120 mV and perfused with 10 µM ACh followed by co-application of 10 µM ACh and 0.1 – 100 µg/mL HAE. Responses were normalised against the peak response of an ACh only control. Mean values ±SEM are plotted and data is fitted with 4 parameter logistic regression curves (GraphPad, Prism 8). (D) IC_{50}s for 1 s data were plotted against V_h and fitted with the Woodhull equation. The slope of the curve (0.16) was significantly greater than 0 ($P = 0.012$) indicating voltage-dependent inhibition by HAE.

Figure 5. **HAE inhibits the ACh response of 3 well characterised mammalian neuronal nAChRs and a hybrid insect/mammalian nAChR expressed in *Xenopus* oocytes.** Representative traces showing the inhibition by 3 µg/mL HAE of ACh-induced currents in the (A) hybrid *Drosophila* α2/chick β2, (B) rat α4β2, (C) human α7 and (D) rat α3β4 nAChRs expressed in *Xenopus* oocytes at V_h = -75 mV.

Figure 6. HAE shows selectivity for the hybrid insect/mammalian receptor and potent action on the late current of the rat α3β4 nAChR. Concentration-inhibition curves showing the actions of HAE on (A) the peak ACh-induced current for the hybrid *Drosophila* α2/chick β2, human α7, rat α3β4 and rat α4β2; and (B) on late current responses for rat α3β4 and rat α4β2 expressed in *Xenopus* oocytes. A 4 parameter logistic regression curve was fitted (GraphPad, Prism 8) from which IC_{50} values were derived. Peak current IC_{50} values of 3.24 µg/mL for α4β2, 2.30 µg/mL for α7, 1.47 µg/mL for α3β4 and 0.440 µg/mL for Dα2/β2 with late current IC_{50} values of 0.431 µg/mL for α4β2 and <<0.03 µg/mL for α3β4 were obtained. All responses were normalised to the control 100/10 µM ACh response and plotted as mean ±SEM (n = 7-8). (C) IC_{50} values for the inhibition of peak current IC_{50} by HAE were plotted as mean ±95 % CI and compared using an extra sum-of-squares F-test (GraphPad, Prism 8). Significant differences were observed in the peak current IC_{50} for Dα2/β2 vs. α7 (P = 0.0102), Dα2/β2 vs. α4β2 (P = 0.0016) and Dα2/β2 vs. α3β4 (P = 0.0217) (* = P<0.05, ** = P<0.01).

Figure 7. **HAE shows voltage-dependent inhibition of rat α4β2, but not rat α3β4 nor human α7 nAChRs.** (A) A voltage ramp was applied to *Xenopus* oocytes expressing rat α4β2 or rat α3β4 nAChRs where the V_h was ramped from -90 mV to +50 mV over 1 s, ACh co-applied with 0.3 ug/mL HAE for α3β4 or ACh co-applied with 3 ug/mL HAE for α4β2 was applied in addition to ACh control (n = 5). When examining the -90 mV to -40 mV range the % control response for α4β2 changed from approximately 44 % to 75 % and for α3β4 the level of inhibition across the same voltage range remained consistent at approximately 25 % of the control response. (B) Responses of human α7 receptors expressed in *Xenopus* oocytes to 100 µM ACh and 100 µM ACh co-applied with 1 µg/mL HAE as the V_h was changed. All points are normalised to the -125 mV ACh response and plotted as mean ±SEM (n = 5). One-way ANOVA showed no significant differences in the percentage of inhibition across the V_h range for each HAE concentration tested.

Figure 8. **HAE shows competitive inhibition of rat α4β2 nAChR but not rat α3β4 nAChR.** Concentration-response curves of ACh alone (black) and ACh co-applied with HAE (red) on (A) rat α3β4 and (B) rat α4β2 nAChRs expressed in *Xenopus* oocytes. 0.3 µg/mL HAE was used for rat α3β4 and 3 µg/mL HAE was used for rat α4β2. EC_{50} values of 161 µM (ACh)/184 µM (ACh + HAE) for α3β4 and 19.9 µM (ACh)/241 µM (ACh + HAE) for α4β2 were obtained. The presence of HAE reduced the top plateau of the dose-response curve to 73.4 % for α3β4 with no effect on α4β2. An extra sum-of-squares F-test showed only the difference in EC_{50} for α4β2 to be statistically significant (P < 0.0001). Each point was normalised to the 1 mM ACh response and plotted as mean ±SEM (n = 5).

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