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Short communication

Galanthamine and other Amaryllidaceae related alkaloids are inhibitors of α7, α4β2 and α3β4 nicotinic acetylcholine receptors

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ABSTRACT

Galanthamine is an Amaryllidaceae-derived acetylcholinesterase inhibitor used to treat memory impairment in Alzheimer's disease and vascular dementia. There is evidence that galanthamine, in addition to its effects on acetylcholinesterase, may enhance or inhibit brain nicotinic acetylcholine receptors, which could increase or decrease the therapeutic efficacy of galanthamine, respectively. Here, we evaluated the effects of galanthamine and two others Amaryllidaceae acetylcholinesterase inhibitors (haemanthamine and tazettine) analyzed by gas chromatography–mass spectrometry and identified by comparing their mass fragmentation patterns with literature and database NIST vs.2.0 on the agonist responses of brain nicotinic acetylcholine receptors α7, α3β4, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$. Using nicotinic acetylcholine receptors expressed heterologously in *Xenopus* oocytes, in conjunction with two-electrode voltage clamping, we found that galanthamine inhibits the function of nicotinic acetylcholine receptors assayed through a mix competitive and non-competitively. Nicotinic acetylcholine receptor α7 were significantly more sensitive to inhibition (17 ± 0.6 μM) than the heteromeric receptor, α3β4 (90 ± 3.4 μM). Neither haemanthamine nor tazettine were more potent than galanthamine.

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Introduction

Galanthamine is a competitive and reversible inhibitor of the enzyme acetylcholinesterase (AChE), used for the treatment of neurodegenerative Alzheimer's disease (AD) (Zarotsky et al., 2003). Traditionally, pharmacological treatments for AD, or similar diseases, related to a deficit of the neurotransmitter acetylcholine (ACh) are aimed to acetylcholinesterase inhibitors (Zarotsky et al., 2003). However, the putative roles for nicotinic acetylcholine receptors (nAChR) in AD has led to search of new candidate AD drugs targeting nAChR from natural or synthetic origin (Arneric et al., 2007). In this regard, in addition to its effects on AChE, electrophysiological studies have suggested that the Amaryllidaceae alkaloid, galanthamine, allosterically enhances the function of a number of nAChR providing therapeutic benefits in the areas of cognition, attention and antineurodegenerative activity (Samochocki et al., 2003). However, other studies using slightly different experimental procedures have found that galanthamine inhibits nAChR in a non-competitive manner (Smulders et al., 2005). The aim of this study was to examine the effects of galanthamine and other Amaryllidaceae alkaloids on the function of α7, α3β4 and the alternate forms of the α4β2 [$(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$] nAChR expressed heterologously in oocytes using electrophysiological.

Material and methods

Chemicals

Galanthamine, haemanthamine and tazettine were previously isolated from of *Rhodolirium andicola* (Poepp.) Traub, synonym of *Rhodophiala andicola* (Poepp.) Traub, Amaryllidaceae, bulbs (Moraga-Nicolás et al., 2018). The plant was identified by Dr. Marcelo Baeza and deposited at the herbarium of Universidad de Concepción, Concepción, Chile (voucher no. CONC 182466). Other chemicals were purchased from Sigma Chemical (Poole, Dorset, UK). Fresh ACh stock solutions were made daily in a oocyte

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perfusion solution (OPS) containing 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4.

Gas Chromatography–Mass Spectrometry (GC/MS) analysis of galanthamine, haemanthamine and tazettine

The well-known Amaryllidaceae alkaloids galanthamine, haemanthamine and tazettine were analyzed by coupled GC-MS with electron impact ionization (70 eV) using an Agilent, model 7890A chromatograph equipped with a HP-5 ms capillary column (30 m by 0.25 mm by 0.25 μm; J&W Scientific) with helium carrier gas. The GC oven was programmed to ramp from 100 °C (for 3 min) to 280 °C at 10 °C/min and held for 19 min. The injector and transfer line temperatures were 250 °C and 285 °C, respectively. The alkaloid compounds were identified by comparing their gas chromatography mass spectra with data from the NIST mass spectrometry Search 2.0 library, Kovats indices (RI) and mass spectra reported in the literature (Ortiz et al., 2012). The Kovats retention indexes of the compounds were recorded with standard of an n-hydrocarbon mixture (C_9-C_{26}). The proportion of each alkaloid in the basic extracts is expressed as a percentage of ion current (TIC).

Nicotinic acetylcholine receptors expression in Xenopus laevis*oocytes*

The α7, α3β4, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ nicotinic acetylcholine receptors (wild type) were expressed heterologously in defolliculated oocytes from *Xenopus laevis*, which were dissected from adult female *X. laevis* frogs (Nasco, USA). The care and use of *X. laevis* frogs in this study was approved by the Oxford Brookes University Animal Research Committee, in accordance with the guidelines of the 1986 Scientific Procedures Act of the United Kingdom. Human α7 cDNA or a mixture of α4 and β2 or α3 and β4 subunit cDNA were injected into the nuclei of oocytes in a volume of 23 nl/oocyte by using a Nanoject Automatic Oocyte Injector (Drummond Scientific, Broomall, PA). For expression of α3β4 receptors the ratio of α3 and β4 cDNA injected was 1:1, whereas for the expression of $(\alpha4)_2(\beta2)_3$ the ratio was 1 α4 to 10 β2 (Moroni and Bermudez, 2006). For expression of $(\alpha4)_3(\beta2)_2$ the ratio used was 10 α4 to 1 β2 (Moroni and Bermudez, 2006). After injection, oocytes were incubated at 17 °C in OPS supplemented with a mixture of penicillin-streptomycin-anphotericin-B (10,000 penicillin, 10 mg streptomycin and 25 μg amphotericin-B/ml) and amikacin (100 μg/ml). Experiments were performed on oocytes 2–6 days after injection.

Electrophysiological recordings

Electrophysiological recording from oocyte post-injection was made at room temperature using a standard two electrode voltage clamp technique with an automatic multichannel system (HiClamp, Multichannel Systems, Germany). Oocytes were impaled by two borosilicate capillary glass (Harvard Instrument: 150 TF GC) microelectrodes filled with 3 M KCl (0.3–2.0 MΩ) and voltage-clamped at −60 mV. During recording, oocyte were perfused OPS, as described in the manual of HiClamp. The sensitivity of the receptors to inhibition by antagonists was tested by first immersing the oocyte into the antagonist for 5 s and then coupling it with an EC_{50} concentration of ACh (100 μM) 10 s. Antagonist concentration-response data were normalized to the appropriate ACh EC_{50}. Between each successive ACh and/or compound application, the cell was perfused with OPS solution for 3 min to allow drug clearance and prevent receptor desensitization. To construct antagonist concentration-effect curves, the responses elicited by co-application of an EC_{50}ACh concentration and increasing concentrations of compound were normalized to the response elicited by an EC_{50} concentration of ACh alone.

Data analyses

Concentration-response data for antagonists were fitted using a nonlinear regression (Prism 5.0; GraphPad, USA). The data were fitted to the logistic equation $Y = Bottom + Top\text{-}Bottom)/(1 + 10^{((LogEC50\text{-}X)*HillSlope)})$, where X is the logarithm of concentration of the antagonist. Y is the response; Y starts at the bottom and goes to Top with sigmoidal shape. Results are presented as mean ± S.E.M. of at least six separate experiments from at least two different batches of oocytes.

Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Data for wild type receptor studied were obtained from oocytes from at least three different donors. Statistical and nonlinear regression analyses of the data from concentration response were performed using Prism 5 (GraphPad, San Diego, CA). Unpaired Student's *t*-tests were used for comparison between two groups (control and test). Values are presented as arithmetic mean ± SEM. Statistical tests with $p < 0.05$ were considered significant.

Results and discussion

Spectroscopic analysis by GC-MS is a valuable tool for the detection, identification and quantification of alkaloids in Amaryllidaceae plants (Cortes et al., 2015). For this study, the technique was used to identify the well-known Amaryllidaceae alkaloids galanthamine, haemanthamine and tazettine comparing their mass spectra with data from the NIST mass spectrometry Search 2.0 library, literature and retention index (Table 1, Fig. 1). The effects of galanthamine and two other related Amaryllidaceae alkaloids, tazettine and haemanthamine, on oocytes expressing heterelogously α7, α3β4, $(\alpha4)_2(\beta2)_3$ or $(\alpha4)_3(\beta2)_2$ were assayed using two electrode voltage clamp recording. Galanthamine inhibited all receptors tested in a concentration-dependent manner with an inhibitory potency (IC_{50}) that was receptor-dependent (Table 2). The rank order of sensitivity to inhibition by galanthamine was: $\alpha7 > (\alpha4)_3(\beta2)_2 \cong (\alpha4)_2(\beta2)_3 \gg \alpha3\beta4$ (see Table 2).

Haemanthamine and tazettine inhibited all nAChR tested but with low potency, compared to galanthamine (Table 2). Galanthamine has been reported to enhance the agonist responses of α4β2, α3β4, α6β4 and α7 nAChR (Samochocki et al., 2003).

Table 1
GC-MS analysis of isolated alkaloids from *Rhodolirium andicola* bulbs.

Alkaloid	[M+] and characteristicions (%)	RI[a]	RIreference	MS reference
Galanthamine	287(90); 286(99); 244(27)	2433	2406[1]	[2][3][4]
Heamanthamine	301 (77), 272 (99), 257(53)	2595	2441[5]	[2] [4]
Tazettine	331(19), 247(71), 240 (20)	2593	2585[6]	[2] [4]

[a] Experimental Kovats index; [NIST]; NIST reference; [1] Berkov et al. (2012); [2] de Andrade et al. (2016); [3] Ortiz et al. (2016); [4] Berkov et al. (2004); [5] de Andrade et al. (2014); [6] Gotti et al. (2006).

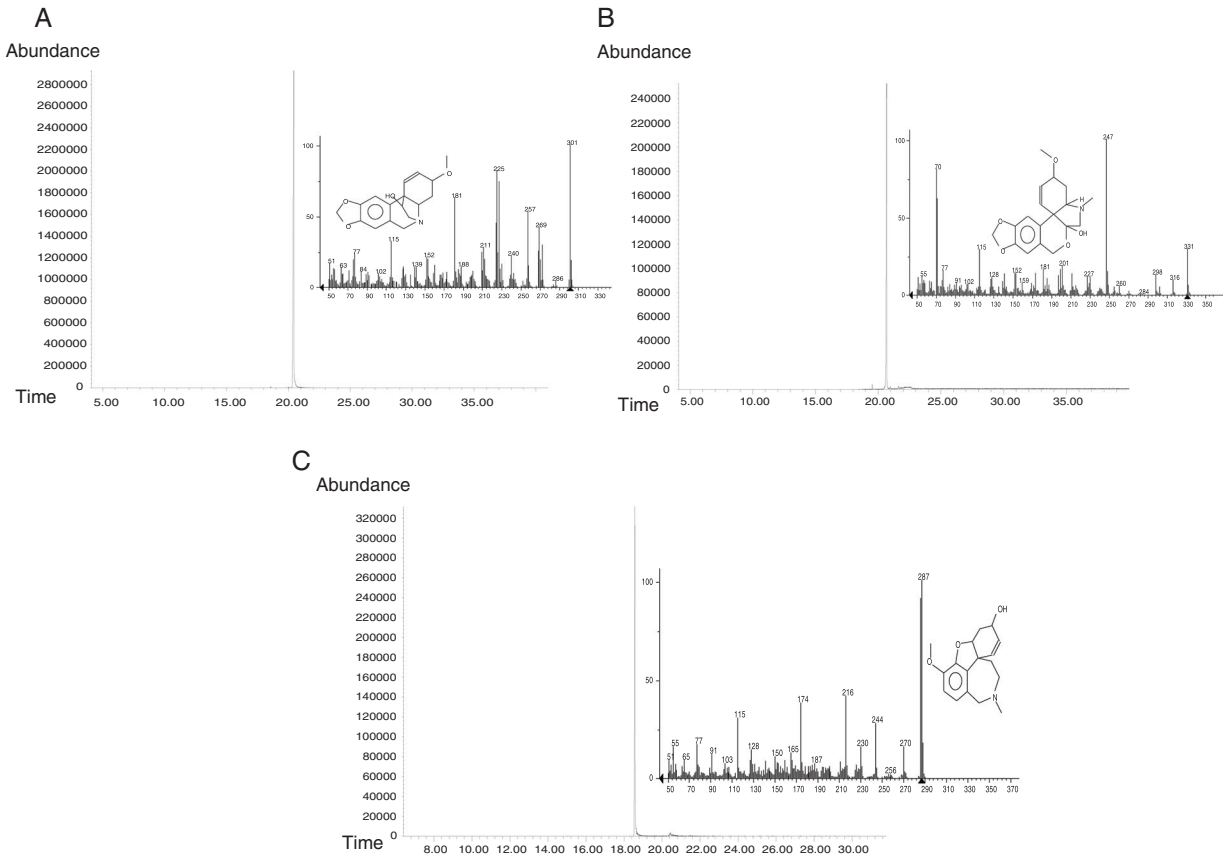

Fig. 1. Chromatograms from GC-MS analisys of haemanthamine (A), tazettine (B) and galanthamine (C), shown estimated purity of isolated compounds.

Table 2
Antagonism of galantahmine and other Amaryllidaceae alkaloids on nicotinic acetylcholine receptors.

Compound	$IC_{50} \pm SEM$ (μM)			
	α7	α3β4	$(\alpha4)_2(\beta2)_3$	$(\alpha4)_3(\beta2)_2$
Galanthamine	17 ± 0.6[c]	90 ± 3.4[c]	24 ± 3.7[c,b]	22 ± 0.6[c,b]
Hemanthamine	97 ± 1.6[c]	216 ± 32[c]	349 ± 7.9[c,d]	246 ± 18[c,d]
Tazettine	276 ± 39[e]	169 ± 12[a]	305 ± 48[a]	367 ± 49[a]

Data represent the mean ± S.E.M. of five experiments.
[a] Levels of significances.
[b] IC_{50} values are not significantly different.
[c] Denotes that the IC_{50} values are all significantly different from each other ($p < 0.0001$).
[d] IC_{50} values between $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ are not significantly different.
[e] Effects of tazettine on heteromeric receptors are significantly different from each other but they are not different from α7 ($p < 0.05$).

Table 3
Effects of galanthamine on the concentration response curve evoked by acetylcholine of nicotinic acetylcholine receptors.

Receptors	Galanthamine–concentration (μM)							
	0		20		40		100	
	EC_{50}	I_{max}	EC_{50}	I_{max}	EC_{50}	I_{max}	EC_{50}	I_{max}
α7	122 ± 6.2[a]	0.9848[b]	129 ± 12[a]	0.8511[b]	132 ± 6.7[a]	0.6773[b]	180 ± 14[a]	0.6404[b]
$(\alpha4)_3(\beta2)_2$	94 ± 1.3[a]	1.107[b]	123 ± 19[a]	0.8313[b]	136 ± 7.1[a]	0.5963[b]	164 ± 3.5[a]	0.3824[b]

The EC_{50} values estimated from the concentration–response curve data are shown as means ± S.E.M.
[a] $p < 0.05$.
[b] $p < 0.0001$, relative to the effects of alkaloid galanthamine.

However, we did not find any potentiating effects for galanthamine, even at concentrations reported to potentiate nAChR. To determine if the inhibitory effects of galanthamine were competitive or non-competitive, we examined the effect of galanthamine on the ACh concentration-response of the receptors α7 and $(\alpha4)_3(\beta2)_2$. As shown Table 3, galanthamine decreased the maximal responses to ACh (I_{max}, which was accompanied by a decrease in ACh potency (EC_{50}). This pattern of inhibition is consistent with a mixed competitive and non-competitive mode of inhibition. This is in contrast to studies that have found that galanthamine enhances agonist

responses of α7 and $(\alpha4)_3(\beta2)_2$ receptors through an allosteric mechanism (Maelicke et al., 2001; Texidó et al., 2005). Interestingly, these studies reported that galanthamine enhanced the agonist responses of nAChR at concentrations ranging from nM (100 nM; Texidó et al., 2005) to μM (0.1–1 μM; Maelicke et al., 2001; Samochocki et al., 2003), concentrations at which we observed inhibition. In agreement with our findings, Smulders et al. (2005) reported that galanthamine inhibits α4β2 nAChR. Furthermore, Kuryatov et al. (2008) found no significant potentiating effects of galanthamine on $(\alpha4)_3(\beta2)_2$ or $(\alpha4)_2(\beta2)_3$ receptors, although they reported that galanthamine appeared to be a specific allosteric modulator of α5α4β2 nAChR. These discrepancies may well reflect differences in experimental conditions. For example, Texidó et al. (2005) tested the effects of galanthamine on the responses of α7 receptors to 500 μM ACh, a concentration that is well above the ACh EC_{50} for human α7 nAChR expressed heterologously in *Xenopus* oocytes (Chavez-Noriega et al., 1997, see also, Table 2) and, critically, observed significant potentiation at only one concentration of galanthamine. Further studies have to be carried out to resolve these discrepancies, however, it is important to note that our studies and those of Smulders et al. (2005) and Kuryatov et al. (2008) were carried out using a wide range of concentrations of galanthamine and that our findings showed that the effects of galanthamine were clearly graded over a wide concentration range.

Does the inhibitory effect of galanthamine on nAChR offset its effects on cholinergic signaling through its effects on AChE? This is unlikely. The IC_{50} for inhibition of AChE by galanthamine is around 0.1–1 μM, whereas inhibition of nAChR occurs at concentrations higher than 10 μM. Indeed, the plasma concentration of galanthamine needed for therapeutic efficacy is of 0.163 ± 0.073, 0.261 ± 0.105 and 0.368 ± 0.145 μmol/l, for different daily doses of 8 mg, 16 mg or 24 mg, respectively (Wattmo et al., 2013) a concentration ten times lower than the concentration of galanthamine required for maximal inhibition of α4β2 nAChR. From this, it is clear that the mild cognitive benefits for patients with Alzheimer's disease are exerted by enhancing cholinergic signaling through inhibition of the enzyme AChE. The cognitive effects of galanthamine are similar to those other cholinesterase inhibitors including donepezil, rivastigmine, and tacrine. In addition, the galanthamine safety profile is similar to that of other cholinesterase inhibitors with regard to cholinergically mediated gastrointestinal symptoms. Indeed, the use of galanthamine in Alzheimer's disease has been approved in several countries, including Argentina, Australia, Canada, Czechia, the European Union (except for The Netherlands), Iceland, Korea, Mexico, Norway, Poland, Singapore, South Africa, Switzerland, Thailand, and the United States (Loy and Schneider, 2006).

Allosteric modulation of α5α4β2 nAChR could still be a therapeutic target for galanthamine, as reported by Kuryatov et al. (2008), making galanthamine a multi-target therapeutic tool.

Conclusions

In conclusion, the results demonstrate that galanthamine and related Amaryllidaceae alkaloids inhibit neuronal nicotinic acetylcholine receptor function. The α7 receptor was significantly more sensitive to inhibition than the heteromeric receptors. We found that galanthamine behaves as a mixed competitive and non-competitive inhibitor of α7 and $(\alpha4)_3(\beta2)_2$receptors. Although several authors have suggested that galanthamine allosterically modulate neuronal nicotinic receptors, our findings revealed that neither galanthamine nor tazettine or haemanthamineallosterically enhanced the function of the nicotinic receptors.

Authors contributions

IB, FM-N, PI-V planned, designed, and executed experimental work. EH and AM performed data analyses. IB, FM-N, wrote the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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