British Journal of Pharmacology (2005) 146, 15–24 © 2005 Nature Publishing Group All rights reserved 0007–1188/05 $30.00
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# Evaluation of benzyltetrahydroisoquinolines as ligands for neuronal nicotinic acetylcholine receptors

[1]Richard Exley, [2]Patricio Iturriaga-Vásquez, [3]Ronald J. Lukas, [4]Emanuele Sher, [2]Bruce K. Cassels & *,[1]Isabel Bermudez

[1]School of Biological and Molecular Sciences, Oxford Brookes University, Gipsy Lane, Oxford OX3 0BP; [2]Millennium Institute for Advanced Studies in Cell Biology and Biotechnology and Department of Chemistry, Faculty of Sciences, University of Chile, Santiago, Chile; [3]Division of Neurobiology, Barrow Neurological Institute, Phoenix, AZ, U.S.A. and [4]Eli Lilly and Co. Lilly Research Centre, Erl Wood Manor, Windlesham, Surrey

**1** Effects of derivatives of coclaurine (C), which mimic the ‘eastern’ or the nonquaternary halves of the alkaloids tetrandrine or *d*-tubocurarine, respectively, both of which are inhibitors of nicotinic acetylcholine receptors (nACh), were examined on recombinant, human $\alpha 7$, $\alpha 4\beta 2$ and $\alpha 4\beta 4$ nACh receptors expressed in *Xenopus* oocytes and clonal cell lines using two-electrode voltage clamping and radioligand binding techniques.

**2** In this limited series, Cs have higher affinity and are most potent at $\alpha 4$ subunit-containing-nACh receptors and least potent at homomeric $\alpha 7$ receptors, and this trend is very marked for the *N*-unsubstituted C and its *O,O′*-bisbenzyl derivative.

**3** 7-*O*-Benzyl-*N*-methylcoclaurine (BBCM) and its 12-*O*-methyl derivative showed the highest affinities and potencies at all three receptor subtypes, and this suggests that lipophilicity at C7 and/or C12 increases potency.

**4** Laudanosine and armepavine (A) were noncompetitive and voltage-dependent inhibitors of $\alpha 7$, $\alpha 4\beta 2$ or $\alpha 4\beta 4$ receptors, but the bulkier C7-benzylated 7BNMC (7-*O*-benzyl-*N*-methylcoclaurine) and 7B12MNMC (7-*O*-benzyl-*N*,12-*O*-dimethyl coclaurine) were voltage-independent, noncompetitive inhibitors of nACh receptors. Voltage-dependence was also lost on going from A to its *N*-ethyl analogue.

**5** These studies suggest that C derivatives may be useful tools for studies characterising the antagonist and ion channel sites on human $\alpha 7$, $\alpha 4\beta 2$ or $\alpha 4\beta 4$ nACh receptors and for revealing structure–function relationships for nACh receptor antagonists.
*British Journal of Pharmacology* (2005) **146,** 15–24. doi:10.1038/sj.bjp.0706307;
published online 27 June 2005

**Keywords:** Tetrandrine; nicotinic acetylcholine receptors; laudanosine; armepavine; *Xenopus* oocytes

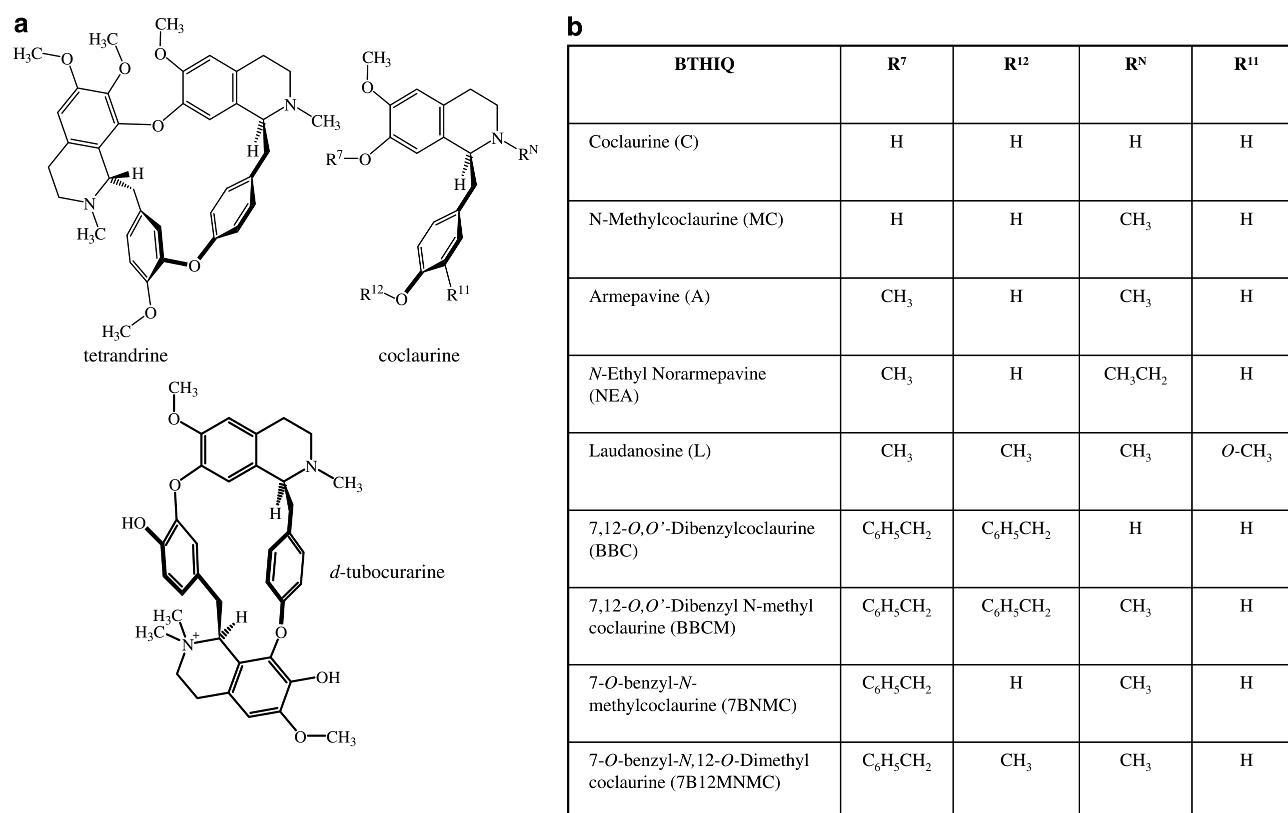
**Abbreviations:** A, armepavine; ACh, acetylcholine; $\alpha$-BgTx, $\alpha$-bungarotoxin; BBC, 7,12-*O,O′*-dibenzylcoclaurine; BBCM, 7,12-*O,O′*-dibenzyl *N*-methyl coclaurine; BBIQ, bis-benzylisoquinoline; 7B12MNMC, 7-*O*-benzyl-*N*,12-*O*-dimethyl coclaurine; 7BNMC, 7-*O*-benzyl-*N*-methylcoclaurine; BTHIQ, 1-benzyl-1,2,3,4-tetrahydroisoquinoline; C, coclaurine; $EC_{50}$, concentration of agonist eliciting a half-maximal response; $IC_{50}$, antagonist concentration eliciting half-maximal inhibition; MC, *N*-methylcoclaurine; nACh, nicotinic acetylcholine; NEA, *N*-ethyl norarmepavine; nHill, Hill coefficient; s.e.m., standard error of the mean

## Introduction

Neuronal nicotinic acetylcholine (nACh) receptors are currently the focus of considerable pharmaceutical interest because of their potential as therapeutic targets for a wide variety of brain diseases such as nicotine addiction, memory and learning disabilities, Parkinson's disease, Tourette's syndrome and Alzheimer's disease (Astles *et al*., 2002). *d*-Tubocurarine, a monoquaternary, head-to-tail bis-tetrahydroisoquinoline alkaloid isolated from curare, is the prototype of an extensive series of natural and synthetic neuromuscular nAChR receptor blockers with activity also at neuronal nACh receptors (Buck, 1987; Garland *et al*., 1998). However, *d*-tubocurarine and its mono- or bisquaternary analogues have awakened little attention as possible neuronal nACh receptor ligands because of their inability to pass the blood–brain barrier. On the other hand, tetrandrine, a nonquaternary head-to-head bis-tetrahydroisoquinoline alkaloid (Figure 1a) that is the principal antihypertensive and muscle relaxant component of the Chinese cardiovascular drug *han fang chi* (*Stephania tetrandra* root) (Wang & Liu, 1985) and that might be expected to reach the brain in pharmacologically active concentrations has also been found quite recently to be a noncompetitive inhibitor of both muscle and neuronal nACh receptors at low micromolar concentrations (Slater *et al*., 2002).

Both *d*-tubocurarine and tetrandrine belong to the large bis-benzylisoquinoline (BBIQ) alkaloid family that includes anti-inflammatory, antiarrhythmic, bactericidal and muscle relaxant alkaloids (Buck, 1987). While *d*-tubocurarine is regarded primarily as a depolarising skeletal muscle relaxant, which may cause hypotension at high doses, the antihypertensive and smooth muscle relaxant properties of tetrandrine are

*Author for correspondence; E-mail: p0054922@brookes.ac.uk




| BTHIQ | $R^7$ | $R^{12}$ | $R^N$ | $R^{11}$ |
|---|---|---|---|---|
| Coclaurine (C) | H | H | H | H |
| N-Methylcoclaurine (MC) | H | H | $CH_3$ | H |
| Armepavine (A) | $CH_3$ | H | $CH_3$ | H |
| *N*-Ethyl Norarmepavine (NEA) | $CH_3$ | H | $CH_3CH_2$ | H |
| Laudanosine (L) | $CH_3$ | $CH_3$ | $CH_3$ | *O*-$CH_3$ |
| 7,12-*O,O'*-Dibenzylcoclaurine (BBC) | $C_6H_5CH_2$ | $C_6H_5CH_2$ | H | H |
| 7,12-*O,O'*-Dibenzyl N-methyl coclaurine (BBCM) | $C_6H_5CH_2$ | $C_6H_5CH_2$ | $CH_3$ | H |
| 7-*O*-benzyl-*N*-methylcoclaurine (7BNMC) | $C_6H_5CH_2$ | H | $CH_3$ | H |
| 7-*O*-benzyl-*N*,12-*O*-Dimethyl coclaurine (7B12MNMC) | $C_6H_5CH_2$ | $CH_3$ | $CH_3$ | H |

**Figure 1** Structure of BTHIQ. (a) Structures of tetrandrine, C ($R^N = R^7 = R^{11} = R^{12} = H$) and derivatives, and *d*-tubocurarine. (b) C and its congeners used in this study.

thought to be a consequence of its ability to inhibit L-type $Ca^{2+}$ channels (King *et al*., 1988; Felix *et al*., 1992). Tetrandrine binds the benzothiazepine site of L-type $Ca^{2+}$ channels and produces the same allosteric coupling pattern as diltiazem (King *et al*., 1988; Felix *et al*., 1992), but its nicotinic effects might contribute to its antihypertensive properties (Slater *et al*., 2002).

The structure of *d*-tubocurarine incorporates two monomeric 1-benzyl-1,2,3,4-tetrahydroisoquinoline (BTHIQ) moieties bonded together in a head-to-tail manner by two ether linkages between the isoquinoline and the benzyl benzene rings (Figure 1a). One of these halves contains a permanently charged, quaternary nitrogen atom, and the other incorporates a tertiary amine function, which may or may not be protonated to create a second positive charge. In contrast, the two halves of the tetrandrine molecule are joined in a head-to-head/tail-to-tail fashion, containing one tertiary nitrogen atom each (Figure 1a). In all BBIQ alkaloids, the two halves may be viewed as derived from the monomeric BTHIQ, coclaurine (C), each with a stereogenic centre. In the case of tetrandrine, as it is usually represented, distinct 'eastern' and 'western' regions are apparent.

The structural features that confer nicotinic activity to tetrandrine have not been determined as yet, partly because the macrocyclic nature of this alkaloid, with very specific bonding between the 'eastern' and 'western' regions of the molecule, hinders the chemical synthesis of a large number of derivatives. However, a recent study has shown that the use of derivatives of C, the monomer that most clearly mimics the structure of the 'eastern' part of tetrandrine (Figure 1a) (and also the nonquaternary half of *d*-tubocurarine), is a valid approach to investigate the structure–functional relationships of tetrandrine analogues at L-type $Ca^{2+}$ channels or noradrenergic receptors (Iturriaga-Vásquez *et al*., 2003). In this study, we have used a similar approach to investigate the structural features of tetrandrine that may confer affinity for neuronal nACh receptors and evaluated the effects of C and *O*- and/or *N*-substituted C derivatives on human $\alpha7$, $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors.

# Methods

## *Chemistry*

C and its *O*-benzylated and/or *O*-methylated derivatives (Figure 1b; BTHIQs will be abbreviated as in Figure 1 henceforward) were prepared by the Bischler-Napieralski 3,4-dihydroisoquinoline synthesis and subsequent reduction of the intermediates with $NaBH_4$. *N*-methylation was carried out on the BTHIQs with aqueous formaldehyde and $NaBH_4$ as previously described (Iturriaga-Vásquez *et al*., 2003).

## *Ligand binding assays*

Established cultures of the SH-SY5Y-h$\alpha$7 clonal cell line (Houlihan *et al*., 2001), which overexpress the human $\alpha7$ nACh receptor, were used for [$^{125}$I]$\alpha$-bungarotoxin ($\alpha$-BgTx) binding assays. SH-EP1-h$\alpha4\beta2$ (Eaton *et al*., 2003) and SH-EP1-h$\alpha4\beta4$ (Eaton *et al*., 2000) clonal cell lines that express human $\alpha4\beta2$

and $\alpha4\beta4$ nACh receptors, respectively, were used for [$^3$H]cytosine binding assays. Membrane homogenates for all clonal cell lines were prepared and utilized in binding assays using methods previously described (Houlihan *et al*., 2001) to give a final protein concentration of 30–50 $\mu$g per assay tube. Competition binding studies were performed in a final volume of 250 $\mu$l of binding saline (in mM: 140 NaCl, 1 EGTA, 10 Hepes, pH 7.4 for [$^{125}$I]$\alpha$-BgTx binding and 120 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 50 Tris, pH 7.0 for [$^3$H]cytisine binding). For [$^{125}$I]$\alpha$-BgTx binding assays, preparations were incubated for 90 min at room temperature (21°C) and the concentration of radiolabelled toxin was 1 nM. In [$^3$H]cytisine binding studies, the concentration of radiolabelled cytisine was 1 nM and incubations were carried out at 4°C for 75 min. For both binding assays, 10 $\mu$M nicotine was used to define nonspecific binding. Bound and free fractions were separated by rapid vacuum filtration through Whatman GF/C filters presoaked in binding saline supplemented with 0.1% polyethyleneimine. Radioactivity was determined with a $\gamma$ counter or by liquid scintillation, as appropriate.

### *nACh receptor expression in* Xenopus *oocytes*

Stage V and VI *Xenopus* oocytes were prepared as previously described (Houlihan *et al*., 2001) and injected nuclearly with PcDNa3.1-h$\alpha$7 or combinations of PcDNA3.1-h$\alpha$4 plus PcDNA3.1h$\beta$2 or PcDNA3.1-h$\beta$4 (1 : 1 molar ratio) containing the indicated, human nACh receptor subunit cDNA using a Nanoject Automatic Oocyte Injector (Drummond, Broomall, PA, U.S.A.). Approximately 1 ng of each plasmid was injected in a total injection volume of 18.4 nl oocyte$^{-1}$. After injection, the oocytes were incubated at 19°C in modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES and 50 $\mu$g ml$^{-1}$ neomycin (pH 7.6 with NaOH). Experiments were performed on oocytes after 2–6 days of incubation (Houlihan *et al*., 2001).

### *Electrophysiological recordings*

Whole-cell currents were measured by two-electrode voltage clamp (GeneClamp 500, Axon Instruments, U.S.A.) using agarose-cushioned electrodes containing 3 M KCl. Oocytes were continually supplied with fresh Ringer solution (in mM: 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 HEPES, pH 7.2) in a 60 $\mu$l bath, using a gravity-driven perfusion system at a rate of 5 ml min$^{-1}$. Modified Ringer solution ($CaCl_2$ replaced by $BaCl_2$) was used when recording from oocytes expressing human $\alpha$7 nACh receptors. Compounds were applied by gravity perfusion using a manually activated valve. The agonist acetylcholine (ACh) was applied for a period sufficient (approx. 10–15 s) to obtain a stable plateau response (at low concentrations) or the beginning of a sag after a peak (at higher concentrations). Between each successive ACh and/or compound application, the cell was perfused with Ringer solution for 3 min to allow drug clearance and prevent receptor desensitisation. Concentration–response curves for ACh were constructed by normalising to the maximal response to ACh and used to generate $EC_{50}$ (concentration of agonist eliciting a half-maximal response) and nHill (Hill coefficient) estimates (Houlihan *et al*., 2001). To construct antagonist concentration–effect curves, the responses elicited by coapplication of an $EC_{50}$ ACh concentration and increasing concentrations of compound were normalised to the responses elicited by an $EC_{50}$ concentration of ACh alone. ACh $EC_{50}$ concentrations at $\alpha$7 and $\alpha4\beta4$ nACh receptors were 100 and 30 $\mu$M, respectively (Houlihan *et al*., 2001; Figures 3b, 6b). The concentration–response curve of ACh at $\alpha4\beta2$ nACh receptors is biphasic comprising a high-affinity ($EC_{50}$ 1 $\mu$M) and a low-affinity component ($EC_{50}$ 100 $\mu$M) (Figure 5b; see also, Zwart & Vijverberg, 1998; Buisson & Bertrand, 2001; Houlihan *et al*., 2001); the human $\alpha4\beta2$ receptor studies reported here were carried out using the low-affinity ACh $EC_{50}$ concentration (100 $\mu$M) due to the predominance of this component (approximately 85% of the overall ACh response; Figure 5b). Constant responses to ACh were obtained before the coapplication of ACh and compound. In these studies, oocytes were preincubated with compounds for 3 min prior to the coapplication procedure to ensure equilibration between receptors and compound. To maintain ongoing measurements of the control response to ACh throughout the experiment, each coapplication was bracketed by an application of $EC_{50}$ of agonist alone.

### *Data analyses*

Concentration–effect data for agonists and antagonists were fitted by nonlinear regression (Prism 3.01, GraphPad, U.S.A.) to the equations:

$$\text{(a) } i = i_{max}/[1 + (EC_{50}/x)^{nHill}] \text{ or}$$

$$\text{(b) } i = i_{max}/[1 + (IC_{50}/x)^{nHill}]$$

wherein $i_{max}$ = maximal normalised current response (in the absence of antagonist for inhibitory currents), $x$ = agonist or antagonist concentration, $EC_{50}$ = concentration of agonist eliciting a half-maximal response, $IC_{50}$ = antagonist concentration eliciting half-maximal inhibition and nHill = Hill coefficient. ACh concentration–response data from $\alpha4\beta2$ receptors were fitted using a two-component Hill equation (Houlihan *et al*., 2001). The magnitudes of the responses to the agonist concentrations greater than 1 mM decreased in a concentration-dependent manner due to receptor desensitisation and/or agonist-induced open channel block and were excluded from the analysis of the data. Results are presented as mean ± standard error of the mean (s.e.m.) of at least four separate experiments from at least two different batches of oocytes. Where appropriate, one-way Anova or Student's *t*-tests for paired or unpaired data were used, and values of $P < 0.05$ were regarded as significant.

The binding parameters ($K_D$ and $B_{max}$) of [$^3$H]cytisine binding were determined from saturation binding isotherm data using the equation $y = (B_{max} \times x)/(K_D + x)$, wherein $B_{max}$ = maximal binding, $K_D$ = apparent equilibrium dissociation binding constant, $x$ = concentration of ligand and $y$ = specific binding.

## Results

The BTHIQ analogues listed in Figure 1b were designed to evaluate the importance of *N*-alkylation and *O*-benzylation or methylation with regard to affinity and selectivity for recombinant, human $\alpha$7, $\alpha4\beta2$ or $\alpha4\beta4$ nACh receptors

expressed heterologously in *Xenopus* oocytes or in transfected clonal cell lines. Each BTHIQ inhibited α7, α4β2 and α4β4 nACh receptor radioligand binding and/or function with a potency and profile that was influenced by the receptor subtype (Tables 1 and 2). None of the ligands tested displayed agonist or potentiating effects (not shown), even at the highest concentrations tested (100–300 μM).

### *α7 nACh receptors*

Binding of $^{125}$I-BgTx to SH-SY5Y-hα7 cell membrane homogenates was inhibited by 7BNMC (7-*O*-benzyl-*N*-methylcoclaurine), 7B12MNMC (7-*O*-benzyl-*N*,12-*O*-dimethyl coclaurine), laudanosine (L), armepavine (A) and *N*-ethyl norarmepavine (NEA) (Figure 2a). In contrast, *N*-methylcoclaurine (MC) and C did not inhibit binding, and BBC (7,12-*O,O′*-dibenzylcoclaurine) or BBCM (7,12-*O,O′*-dibenzyl *N*-methyl coclaurine) caused less than 10% inhibition at the highest concentration tested (300 μM) (Figure 2a). Estimated $IC_{50}$ values are summarised in Table 1. The rank order of potency of inhibition of $^{125}$I-BgTx binding to SH-SY5Y-hα7 cell α7 nACh receptors was 7BNMC > 7B12MNMC > L > A > NEA ≫ BBC ≈ BBCM. Characterisation of the effects of the most potent inhibitory BTHIQs at concentrations close to their $IC_{50}$ concentration on the saturation binding of $^{125}$I-BgTx showed that they are noncompetitive inhibitors of human α7 nACh receptors: A, L, NEA, 7BNMC and 7B12MNMC significantly decreased $B_{max}$ from 1.55 pmol mg protein$^{-1}$ to about 0.8–0.9 pmol mg protein$^{-1}$ ($P < 0.05$) without significantly changing $K_D$ values. Control $K_D$ was 0.98 nM and in the presence of BTHIQ increased to no more than 1.2 nM (Figure 2b).

$EC_{50}$ ACh-mediated currents through α7 nACh receptors expressed heterologously in *Xenopus* oocytes were fully inhibited by 7BNMC, 7B12MNMC, A, L and NEA, with $IC_{50}$ (antagonist concentration eliciting half-maximal inhibition) values of 1.4 and 2.8 μM for 7BNMC and 7B12MNNC and 26 and 22 μM for A and L (Figure 3a; Table 2). Inhibition occurred at concentrations similar to or lower than those producing inhibition of radiotoxin binding. NEA, the *N*-ethylated analogue of A, was significantly less potent

**Table 1** Ligand binding affinities ($IC_{50}$ (μM); mean (95% CI); 4–5 independent experiments) for BTHIQ compounds at human α4β2 or α4β4 nACh receptors expressed in SH-EP1 cells or α7 nACh receptors expressed in SH-SY5Y cells

| | *IC₅₀ (95% CI)* (μM) | | |
|---|---|---|---|
| | *α7* | *α4β2* | *α4β4* |
| BTHIQ | [$^{125}$I]α-BgTx | [$^{3}$H]cytisine | [$^{3}$H]cytisine |
| C | ≥500 | >200 | 132 (125–140) |
| MC | ≥500 | 27 (18–38) | 23 (19–27) |
| A | 28 (13–59) | 24 (13–47) | 14 (9–24) |
| NEA | 44 (38–92) | 59 (38–92) | 47 (37–60) |
| L | 21 (11–41) | 16 (13–19) | 12 (10–16) |
| BBC | >300 | 13 (8–21) | 7.8 (6.0–10) |
| BBCM | >300 | 15 (11–22) | 7.3 (6.1–21.7) |
| 7BNMC | 11 (9–13) | 1.4 (1.2–1.7) | 0.37 (0.26–0.52) |
| 7B12MNMC | 14 (11–18) | 8.1 (7.0–9.5) | 2.6 (2.1–3.1) |

In competition studies using α7-, α4β2 or α4β4 nACh receptors, the radiolabelled ligand concentration was 1 nM.

**Table 2** Functional affinities ($IC_{50}$ (μM); mean (95% CI); 6–10 independent experiments) for BTHIQ compounds at human α7, α4β2 or α4β4 nACh receptors expressed heterologously in *Xenopus* oocytes

| | *IC₅₀ (95% CI)* (μM) | | |
|---|---|---|---|
| | *α7* | *α4β2* | *α4β4* |
| BTHIQ | ACh $EC_{50}$ 100 μM | ACh $EC_{50}$ 100 μM | ACh $EC_{50}$ 30 μM |
| C | >200 | 49 (23–100) | 18 (15–23) |
| A | 25 (19–36) | 13 (9–18) | 4.8 (3.9–5.8) |
| NEA | 43 (34–54) | 21 (15–28) | 17 (14–19) |
| L | 22 (16–29) | 8.6 (5.8–12.8) | 3.3 (2.0–5.5) |
| BBC | >200 | 9.7 (6.9–13.6) | 2.2 (1.5–3.1) |
| 7BNMC | 1.4 (1.2–1.5) | 0.97 (0.72–1.31) | 0.24 (0.23–0.26) |
| 7B12MNMC | 2.8 (2.3–3.3) | 4.0 (3.4–4.7) | 1.2 (0.8–1.7) |

Data represent 6–10 independent experiments.

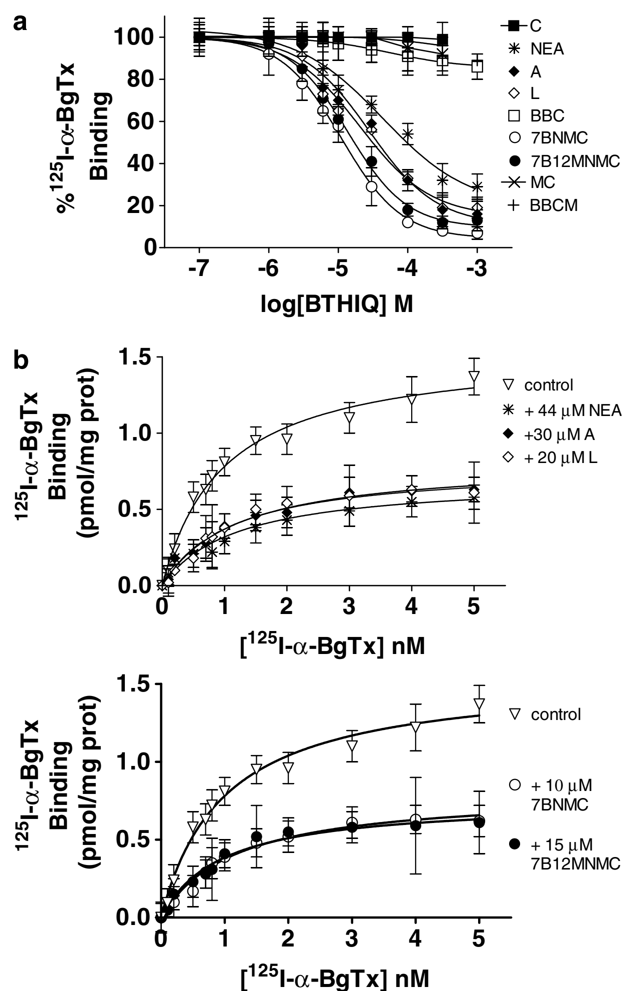



**Figure 2** Effects of BTHIQs on binding of [$^{125}$I]α-BgTx to SH-SY5Y-hα7 membrane homogenates. (a) Displacement of [$^{125}$I]α-BgTx binding to SH-SY5Y-hα7 cells by BTHIQ. SH-SY5Y-hα7-membrane homogenates were incubated with 1 nM [$^{125}$I]α-BgTx for 90 min at room temperature in the presence of various concentrations of BTHIQ. Data are the mean ± s.e.m. of 4–5 experiments. (b) Saturation analysis of the specific binding of [$^{125}$I]α-BgTx to SH-SY5Y-hα7-membrane homogenates in the absence and presence of $IC_{50}$ concentrations of BTHIQ. Data points are the means of triplicate samples ± s.e.m. of eight experiments.

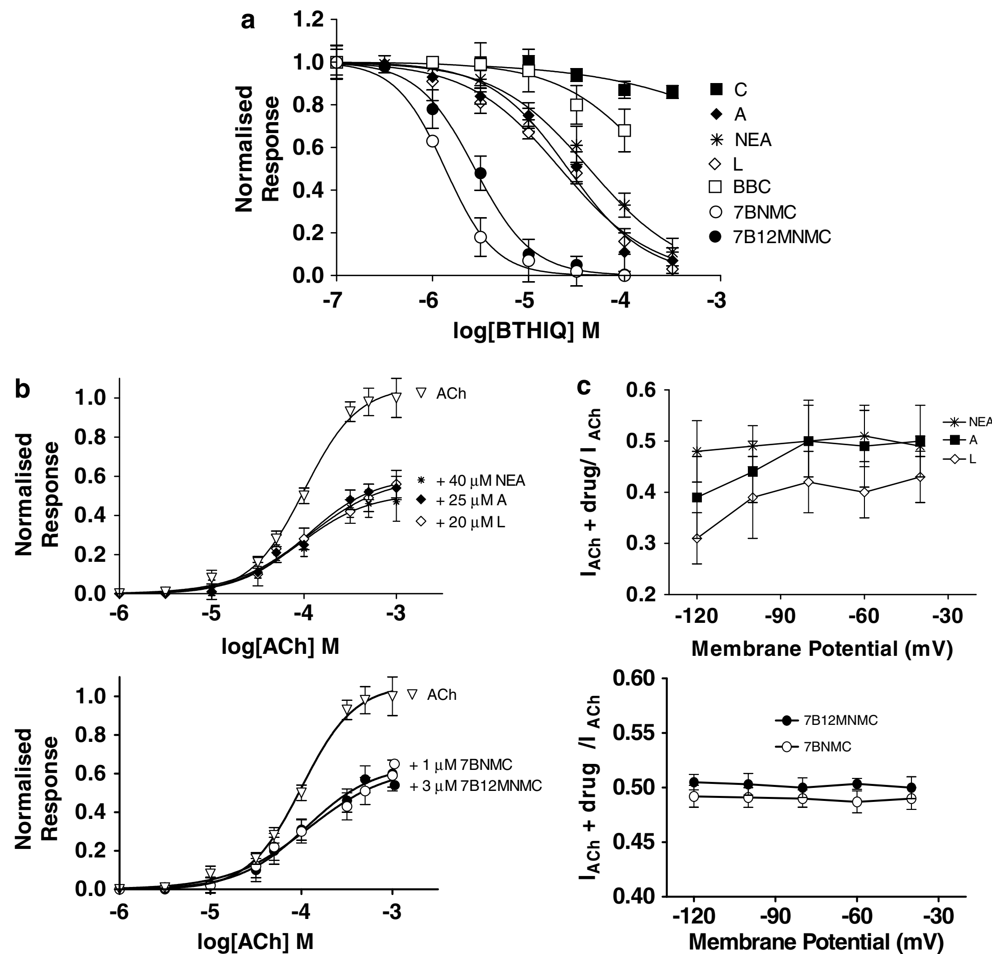



**Figure 3** Functional effects of BTHIQs on human α7 nACh receptors. (a) Concentration–response curve for antagonist effects of the indicated BTHIQs on function of human α7 nACh receptors. The data were normalised to the responses elicited by 100 $\mu$M ACh (approx. $EC_{50}$ of ACh at α7 nACh receptors) and then fitted to a single site Hill equation. Data points represent the mean ± s.e.m. of 6–10 experiments. Where no error bars are shown, they are smaller than the symbols. (b) Concentration–response curve for ACh responses in the absence or presence of $IC_{50}$ concentrations of BTHIQ. Oocytes were first exposed to ACh to obtain control responses, then to BTHIQ for 2 min, and finally to both ACh and BTHIQ. Data were normalised to responses elicited by 1 mM ACh (maximal ACh response) and represent the mean ± s.e.m. of 8–10 experiments. (c) Data show the inhibition of α7 receptor function by concentrations of BTHIQ close to their respective $IC_{50}$ values at a range of membrane potentials. Inhibition by NEA, 7BNMC and 7B12MNMC was equivalent at all potentials, but inhibition by L or A was dependent on holding potential.

($IC_{50}$ = 43 $\mu$M; $P < 0.05$) than any of the *N*-methylated BTHIQs tested (MC and BBCM were not tested). *N*-unsubstituted BTHIQ (C and BBC) had weaker effects on the function of α7 nACh receptors. C, at 300 $\mu$M, inhibited less than 10% of the ACh $EC_{50}$ response, while 100 $\mu$M BBC inhibited approximately 30% of the ACh $EC_{50}$ response (Figure 3a). The rank order of potency of inhibition of human α7 receptors by BTHIQs is 7BNMC > 7B12MNMC > L ≌ A > NEA ≫ BBC > C.

The most potent inhibitors of α7 receptor function (7BNMC, 7B12MNMC, L, A, NEA) were selected to investigate how BTHIQs affect the concentration–response curve of ACh at α7 receptors. These studies were carried out using concentrations of BTHIQs close to their functional $IC_{50}$ concentrations. Figure 3b shows that all the BTHIQ compounds tested significantly lowered ACh efficacy throughout the agonist dose range by about 40–50% ($P < 0.05$) without substantial increases in the ACh $EC_{50}$. In a further series of experiments, $EC_{50}$ concentrations of ACh for α7 (100 $\mu$M) were used to determine whether the antagonism by 7BNMC, 7B12MNMC, L, A or NEA was voltage-dependent. For these experiments, oocytes were stepped at 20 mV intervals between −100 and −40 mV and the response to ACh was determined at each potential in the presence and absence of antagonist. The percentage of inhibition for NEA, 7BNMC and 7B12MNMC was not significantly different at each holding potential (Figure 3c). In contrast, the effects of A and L were significantly more pronounced at voltages lower than −80 mV (Figure 3c; ($P < 0.05$). These data indicate that BTHIQs inhibited α7 nACh receptors noncompetitively and confirm the findings of Chiodini *et al*. (2001) that L blocks α7 nACh receptors in a voltage-dependent manner.

### *α4β2 and α4β4 nACh receptors*

Binding of [$^3$H]cytisine to both human α4β2 and α4β4 nACh receptors was inhibited by all BTHIQs shown on Figure 1 (Figure 4a). At both receptor subtypes, the most potent inhibitors were 7BNMC and 7B12MNMC, which inhibited

binding at low micromolar levels (Table 1). MC fully inhibited binding of [$^3$H]cytisine to $\alpha4\beta2$ or $\alpha4\beta4$ nACh receptors with potencies significantly higher than that displayed by C at either receptor subtype ($P<0.05$; Table 1). The potency of BBCM at $\alpha4\beta2$ or $\alpha4\beta4$ nACh receptors was not significantly different from that of BBC. Inhibition of [$^3$H]cytisine binding to both receptor subtypes occurred at concentrations similar to or higher than those producing functional inhibition, and they all decreased maximal binding from about 5 pmol mg protein$^{-1}$ to about 3–3.5 pmol mg protein$^{-1}$ without any significant increase in $K_D$ values (Figure 4b), indicating that BTHIQs inhibited binding of [$^3$H]cytisine to human $\alpha4\beta2$ or $\alpha4\beta4$ nACh receptors by a noncompetitive mechanism.

The function of human $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors was fully inhibited by 7BNMC, 7B12MNMC, BBC, L, A, NEA and C (MC and BBCM were not tested), generally with $IC_{50}$ values significantly lower than those observed at $\alpha7$ nACh receptors ($P<0.05$; Table 2). Thus, C and BBC, which had little effect on the function of $\alpha7$ nACh receptors, fully inhibited the ACh responses of $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors (Figures 5a, 6a) with $IC_{50}$ values ranging from low to moderate micromolar concentrations (Table 2). BTHIQs were more potent at inhibiting the function of $\alpha4\beta4$ than that of $\alpha4\beta2$ nACh receptors, but in both receptor subtypes the rank order of potency of functional inhibition was 7BNMC$>$ 7B12MNMC$>$BBC$\simeq$L$>$A$>$NEA$\simeq$C.

To study further the mechanism whereby 7BNMC, 7B12MNMC, BBC, L, A, NEA and C inhibit human $\alpha4\beta2$ or $\alpha4\beta4$ nACh receptors, we analysed the effects of $IC_{50}$ concentrations on membrane currents elicited by different ACh concentrations applied to human $\alpha4\beta2$ or $\alpha4\beta4$ nACh receptors. Figures 5b and 6b show that all the BTHIQ compounds tested significantly reduced the responses to ACh equieffectively for both $\alpha4\beta2$ or $\alpha4\beta4$ nACh ($P<0.05$), without significant changes in the ACh $EC_{50}$. 7BNMC, 7B12MNMC, NEA, C and BBC blocked the response to ACh at human $\alpha4\beta2$ or $\alpha4\beta4$ nACh receptors in a voltage-independent manner (Figures 5c, 6c). However, human $\alpha4\beta2$ or $\alpha4\beta4$ nACh receptors, as human $\alpha7$ nACh receptors, were inhibited in a voltage-dependent manner by A and L (Figures 5c and 6c).

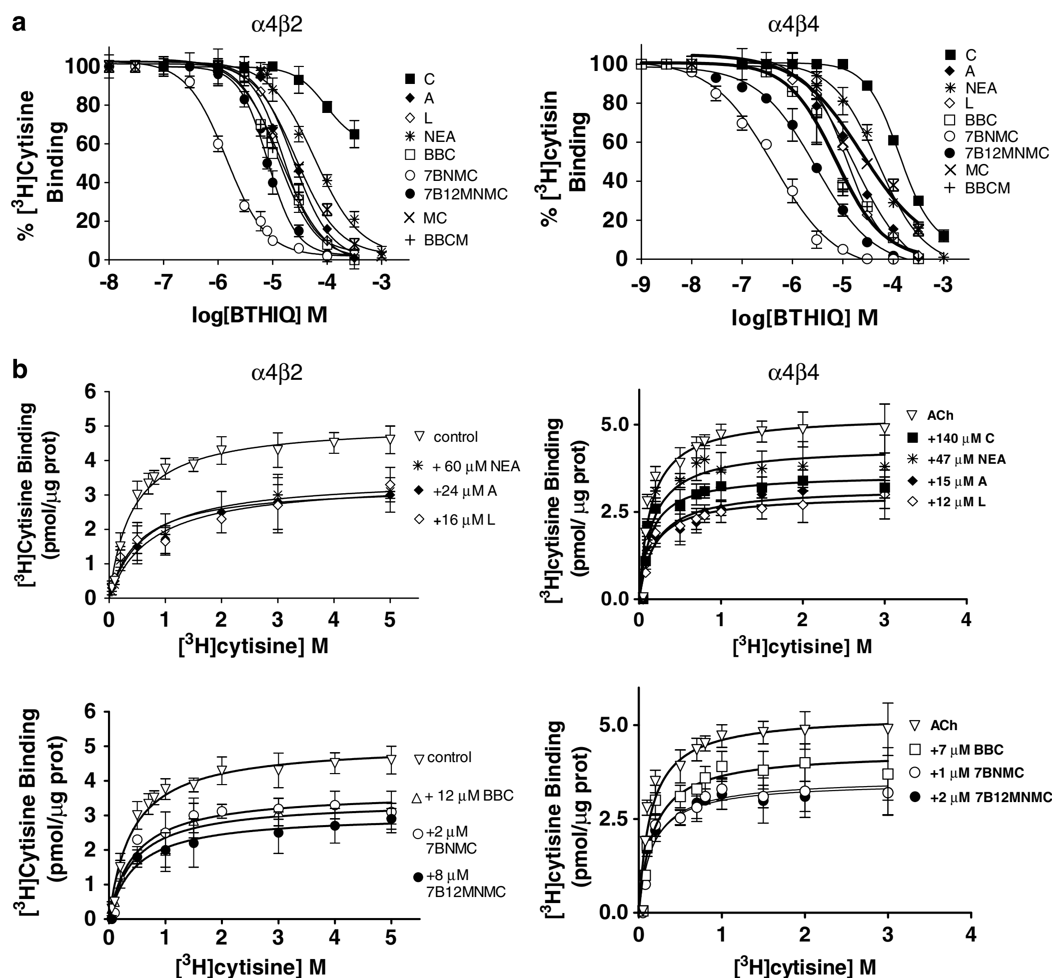



**Figure 4** Effects of BTHIQ on [$^3$H]cytisine binding to human $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors. (a) Displacement of [$^3$H]cytisine binding to SHEP-h$\alpha4\beta2$ or SHEP-h$\alpha4\beta4$ membrane homogenates by BTHIQ. SH-EP1-h$\alpha4\beta2$ or SHEP-h$\alpha4\beta4$ membrane homogenates were incubated with 1 nM [$^3$H]cytisine for 75 min at 4°C in the presence of various concentrations of BTHIQ. Data are the mean$\pm$s.e.m. of 10 experiments. (b) Saturation analysis of the specific binding of [$^3$H]cytisine to SH-EP1-h$\alpha4\beta2$ or SHEP-h$\alpha4\beta4$ membrane homogenates in the absence and presence of $IC_{50}$ concentrations of BTHIQ. Data points are the means of triplicate samples$\pm$s.e.m. of 10 ($\alpha4\beta2$) or 4–5 ($\alpha4\beta4$) experiments.

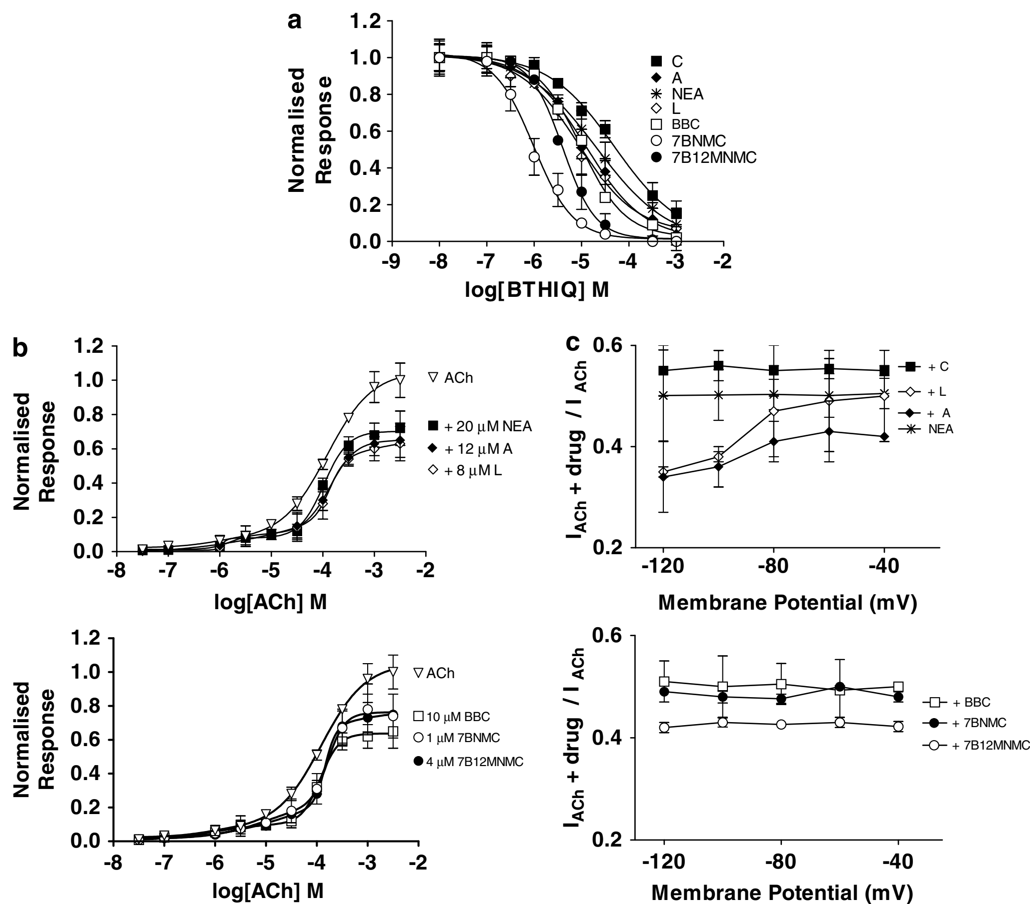



**Figure 5** Functional effects of BTHIQ on human $\alpha4\beta2$ nACh receptors. (a) Concentration–response curve for the antagonist effects of the test BTHIQ on human $\alpha4\beta2$ nACh receptors. The data were normalised to the responses elicited by 100 μM ACh (approximate low affinity $EC_{50}$ of ACh at $\alpha4\beta2$ nACh receptors) and then fitted to a two-component Hill equation. Data points represent the mean ± s.e.m. of 8–10 independent experiments. Where no error bars are shown, they are smaller than the symbols. (b) Concentration–response curve for ACh responses in the absence or presence of $IC_{50}$ concentrations of BTHIQ. After control $EC_{50}$ ACh responses were elicited, oocytes were first superfused with BTHIQ alone for 2 min and then with $EC_{50}$ ACh and BTHIQ. Data were normalised to responses elicited by 1 mM acetylcholine (maximal ACh response) and represent the mean ± s.e.m. of 10 experiments. (c) Inhibition of $\alpha4\beta2$ receptor function by $IC_{50}$ concentrations of BTHIQ at a range of holding potentials. Inhibition by 7BNMC, 7B12MNMC, BBC, NEA and C was equivalent at all potentials, but inhibition by L or A was dependent on holding potential ($n = 10$, Anova test).

## Discussion

The results presented here show that C and its congeners listed in Figure 1 inhibit human $\alpha7$, $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors in a noncompetitive manner and with different strengths. The BTHIQs tested in this study are structural mimics of the 'eastern' moiety of the hypotensive alkaloid tetrandrine, which inhibits muscle and neuronal nAChR with low micromolar affinity (Slater *et al*., 2002). Substitutions at C7, C12 or *N* of the basic BTHIQ structure (Figure 1) produced derivatives that were more potent than C and in some cases (e.g. 7BNMC) more potent than tetrandrine.

C derivatives also mimic the nonquaternary half of the *d*-tubocurarine molecule. The latter has a broad range of pharmacological effects on neuronal nACh receptors, including competitive inhibition (Lipscombe & Rang, 1987; Bertrand *et al*., 1990; Chavez-Noriega *et al*., 1997), partial agonism (Nooney *et al*., 1992; Cachelin & Rust, 1994) and competitive potentiation (Cachelin & Rust, 1994). However, unlike *d*-tubocurarine, BTHIQs only exhibited noncompetitive (voltage-dependent or -independent) inhibitory effects. This suggests, as has been previously shown for muscle nACh receptors (Codding & James, 1973), that two appropriately spaced positively charged nitrogen atoms borne on a rigid hydrocarbon scaffolding fulfil the basic requirement for curariform competitive antagonism at neuronal nACh receptors.

In comparing functional $IC_{50}$ values to radioligand binding inhibition $IC_{50}$ values for these noncompetitive interactions acting at a specific nACh receptor subtype, BBC was four-fold functionally less potent, A, L and NEA were approximately equipotent, and 7BNMC and 7B12MNMC were 5-8-fold functionally more potent when acting at $\alpha7$ nACh receptors, suggesting possible ability of these agents to discriminate sites for radiotoxin binding from functionally relevant agonist binding sites. However, all BTHIQs were slightly more potent in functional than in radioagonist binding competition assays when acting at $\alpha4\beta2$ (1.5–2.8-fold) and $\alpha4\beta4$ (1.5–3.8-fold

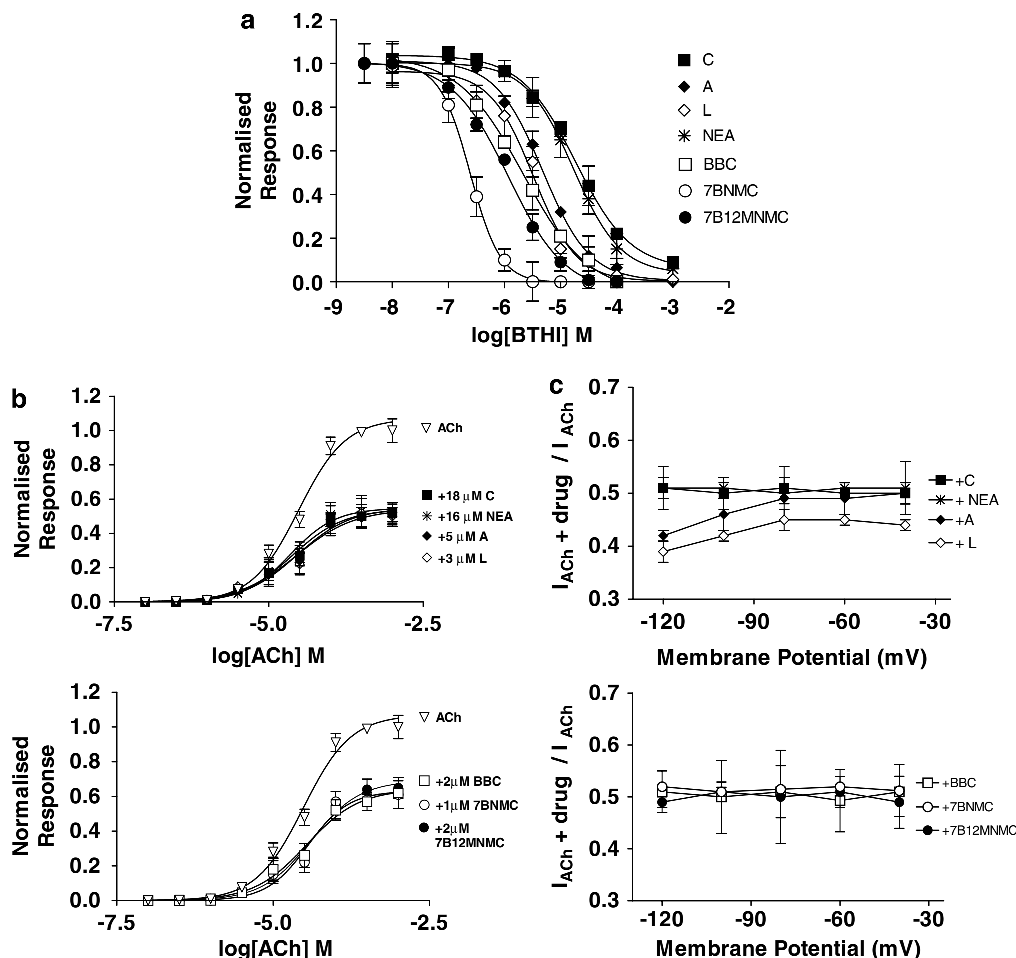



**Figure 6** Functional effects of BTHIQ on human $\alpha4\beta4$ nACh receptors. (a) Concentration–response curve for the antagonist effects of the test BTHIQ on human $\alpha4\beta4$ nACh ceptors. The data were normalised to the responses elicited by 30 $\mu$M ACh (approximate $EC_{50}$ of the ACh response at $\alpha4\beta4$ nACh receptors) and then fitted to a single component Hill equation. Data points represent the mean$\pm$s.e.m. of 3–4 experiments. Error bars are not shown when they are smaller than the symbols. (b) Concentration–response curve for ACh responses in the absence or presence of $IC_{50}$ concentrations of BTHIQ. After control $EC_{50}$ ACh responses were elicited, oocytes were first superfused with BTHIQ alone for 2 min and then with $EC_{50}$ ACh and BTHIQ. Data were normalised to responses elicited by 1 mM ACh (maximal ACh response) and represent the mean$\pm$s.e.m. of 6–10 experiments. (c) Inhibition of $\alpha4\beta4$ receptor function by concentrations of BTHIQ close to their respective $IC_{50}$ concentrations at a range of holding potentials. Inhibition by 7BNMC, 7B12MNMC, BBC, C and NEA was equivalent at all potentials at which ACh responses could be elicited, but inhibition by L or A was dependent on holding potential ($n=10$, Anova test).

excluding C) nACh receptors, possibly suggesting a systematic difference in affinity determinations based on the two assays probing effects on agonist binding domains. In absolute terms, each BTHIQ was most potent at $\alpha4\beta4$ nAChRs and least potent (except functionally for 7B12MNMC and in binding assays for NEA) at $\alpha7$ nAChRs.

What are the key structural features of C and its congeners that influence potency in antagonism of nACh receptors? From the data shown in Tables 1 and 2 it is clear that 7BNMC and 7B12MNMC are the most potent ligands at human $\alpha7$, $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors. These compounds differ from C7-hydroxyl, C12-hydroxyl, *N*-unsubstituted C in that they are *N*-methylated and contain a bulky benzyloxy group at C7 and a phenolic hydroxyl (7BNMC) or methoxyl (7B12MNMC) group at C12. Simpler *N*-methylated Cs contain a hydroxyl (MC) or methoxyl (A, NEA, L) group at C7 and either a hydroxyl (MC, A, NEA) or methoxyl (L) group at C12.

A large, lipophilic substituent at C7 of BTHIQs, which corresponds to part of the 'western' tetrahydroisoquinoline moiety of tetrandrine, is an important element for activity at nACh receptors. Lipophilic substituents at C7 may enhance binding of the ligand to a lipophilic region at or around the BTHIQ binding domain, which may contribute favourable hydrophobic interactions to the free energy of BTHIQ binding to the receptors. Nevertheless, the overall bulkiness in the region also is important. A large lipophilic substituent at C7 such as a benzyloxy group favours interaction with $\alpha7$, $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors more than a small group such as a methoxy group (e.g., 7BNMC is more potent than A), and compounds with a C7-methoxyl group also are generally more potent than hydroxyl analogues (e.g., A is more potent than MC except at $\alpha4\beta2$-nACh receptors). However, the presence of bulky benzyloxy substituents at both C12 and C7 (i.e., BBC and BBCM) decreases potency relative to the potency displayed by 7BNMC or 7B12MNMC, and C12-hydroxylated

7BNMC has higher potency than C12-methoxylated 7B12MNMC for compounds already carrying C7-benzyloxy groups. Such a decrease in potency does not occur in L, which is methoxylated at both C12 and C7, when compared to A. Thus, although lipophilicity on the 'western' side of BTHIQs increases potency, excessive bulkiness may distort the folding of the BTHIQs and weaken their interaction with nACh receptors.

Effects of *N*-alkylation of BTHIQs on affinity for nACh receptors are influenced by the type of alkyl substituent and crucially by receptor subtype. *N*-unsubstituted BTHIQ (i.e., C and BBC) are poor functional antagonists ($IC_{50}$ values in millimolar range) of $\alpha$7 nACh receptors, but the *N*-methylated A, L, 7BNMC and 7B12MNMC inhibit function and binding with micromolar potency. NEA, which is the *N*-ethylated analogue of A, is slightly less potent than A, but it is still significantly more potent than C and BBC. On the other hand, *N*-methylation of C to MC or of BBC to BBCM does not improve or diminishes ability of the compounds to inhibit $^{125}$I-BgTx binding to $\alpha$7 nACh receptors. Thus, BTHIQ activity at $\alpha$7 nAChR seems to be mostly influenced by the type of substituents at C7 and C12: a bulky lipophilic group at C7 conferring highest potency.

In contrast, although BTHIQ activity at $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors does not require *N*-alkylation, this structural modification increases affinity assessed using binding assays when imposed on C. NEA, however, is the least potent of the *N*-alkylated BTHIQs, and its interactions with $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors (and with $\alpha$7 receptors) may be weakened (compared to A) by the larger bulk of the *N*-ethyl group. Moreover, the effect of *N*-methylation on potency when acting at $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors is diminished when other structural requirements such as appropriate bulk on the 'western' side of the BTHIQs are met: BBCM and BBC have comparable affinities for [$^3$H]cytisine binding to $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors. Thus, *N*-methylation and the overall bulk of substituents at C7 and C12 interact to influence BTHIQs' activity at $\alpha4\beta2$ and $\alpha4\beta4$ nACh receptors.

Inhibition of nACh receptors by A or L was voltage dependent. Surprisingly, NEA, which is an *N*-ethylated analog of A, blocked nACh receptors in a voltage-independent manner. This loss of voltage-dependence may be related to steric hindrance of a key interaction between the nitrogen atom and a site at or within the ion channel. Voltage-dependence is also lost with increasing bulk: benzylated *N*-methylated BTHIQs (7BNMC and 7B12MNMC) inhibited nACh receptors in a voltage-independent manner, which suggests that in addition to accessibility to the substituted nitrogen atom, bulk and/or lipophilicity at C7 also influence the ability of BTHIQ to interfere with voltage sensing by nACh receptors.

Our results show that subtle chemical modifications to the basic BTHIQ structure bring about significant changes in both nACh receptor affinity and mode of inhibition. The impact of the structural changes upon affinity is significantly influenced by receptor subunit composition, which further highlights the potential of nicotinic antagonists in the development of high affinity, receptor subtype-specific probes as tools to enhance the study of the roles of nACh receptors in both normal brain functions and in disease. Moreover, BTHIQs have potential for revealing structure–function relationships for nACh receptor antagonists.

This work was funded in part by a Wellcome Trust CRIG and ICM Grant No. P99-031-F. B.K.C. acknowledges a generous gift of equipment from the Alexander von Humboldt Foundation (Germany). R. Exley was funded by a BBSRC-Eli Lilly Case PhD studentship. Funding from National Institutes of Health grants NS40417 and DA015389, from Arizona Disease Control Research Commission grants 9730 and 9615, and by endowment and/or capitalization funds from the Men's and Women's Boards of the Barrow Neurological Foundation also is acknowledged (R.J.L.).

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*(Received February 1, 2005*
*Revised March 24, 2005*
*Accepted May 16, 2005*
*Published online 27 June 2005)*