JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2003; **46**: 1117–1125.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jlcr.744

Research Article

Synthesis and evaluation of tritium labelled 10-methylgalanthamine iodide: a novel compound to examine the mechanism of interaction of galanthamine derivatives with the nicotinic acetylcholine receptors

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Summary

A new promising galanthamine derivative, 10-[^{3}H]methylgalanthamine iodide, was synthesized for binding studies to nicotinic acetylcholine receptors expressed in Torpedo electric ray electroplaques. Galanthamine was reacted with [^{3}H]methyl iodide to yield 10-[^{3}H]methylgalanthamine iodide with a radiochemical yield of $>70\%$ and a specific activity of 32 Ci/mmol after purification via solid phase extraction. To test the ligand properties of the radioligand, calcium imaging and electrophysiology of the non-radioactive analogue were performed to obtain an EC_{50} of 270 nM, a Hill coefficient of 1.9 and the induced cell current. Copyright © 2003 John Wiley & Sons, Ltd.

Key Words: nicotinic acetylcholine receptor (nAChR); acetylcholinesterase inhibitor (AChE-I); allosterically potentiating ligand (APL); galanthamine; tritium

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Received 12 May 2003
Revised 9 June 2003
Accepted 24 June 2003

Introduction

Galanthamine (1) belongs to a new class of non-competitive nicotinic acetylcholine receptor agonists which act as allosterically potentiating ligands (APL).[1] As APLs they can enhance the sensitivity of the receptor towards acetylcholine (ACh), resulting in improved nicotinic cholinergic neurotransmission. The binding site of APLs was found to reside within the extracellular part of the α-subunit of nAChR Torpedo electrocytes (cf. Figure 2) and the mechanism of action of these ligands was clarified.[2,3] The structure–activity relationship of APLs is not yet fully understood because most of them interact with additional target molecules in the mammalian brain. Galanthamine for example is not only an APL, but also an acetylcholinesterase inhibitor (AChE-I). From a medical standpoint, APL can intensify the release of different neurotransmitters via a receptor-mediated mechanism. [^{3}H]Tritium labelling of galanthamine has previously been reported.[4] Because of its higher polarity, 10-methylgalanthamine iodide is less likely to partition into the membrane than galanthamine itself and it is thus particularly useful for *in vitro* applications. The tritiated 10-methylgalanthamine iodide (2) was therefore synthesized for a binding assay using torpedo-fish membrane fragments and the α4 nAChR subtype according to an already established method[5] (Figure 1).

Results and discussion

The non-radioactive standard compound 10-methylgalanthamine iodide, which was needed for analytical purposes such as HPLC, was

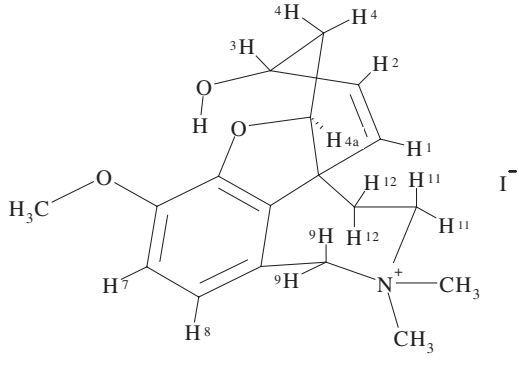

Figure 1. Structure of 10-methylgalanthamine iodide

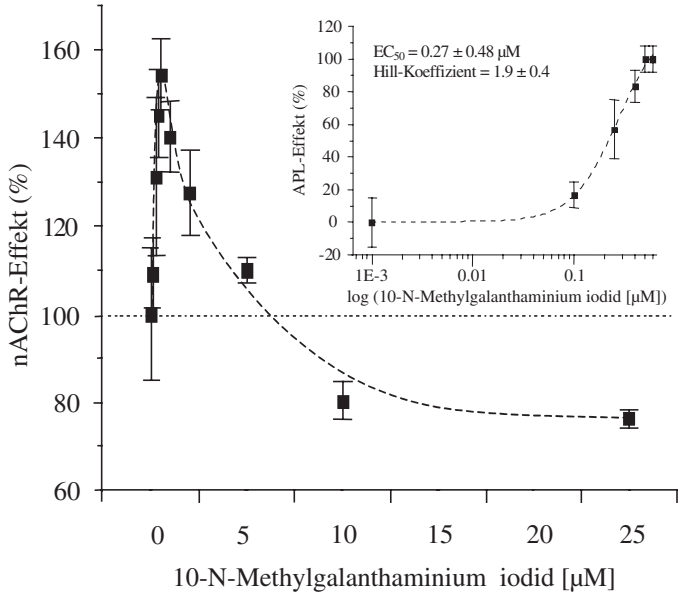

Figure 2. Effect of 10-methylgalanthamine iodide on the dose–response curve for nicotine-activated Ca^{2+} influx into HEK-293 cells expressing the human $\alpha4\beta2$ nAChR subtype

synthesized by a substitution reaction of methyl iodide and galanthamine in yields of 80%.[6] Several *in vitro* studies were performed to evaluate the pharmacological properties of 10-methylgalanthamine iodide. Galanthamine and 10-methylgalanthamine iodide were compared according to their APL effect. Under conditions of the standard calcium imaging assay, galanthamine did not produce any electric signals in HEK-293 cells expressing the $\alpha4\beta2$ subtype of nAChR, probably because it largely partitioned into the cell membranes instead of binding to the receptor and activated its integral cation channel. However, when 10-methylgalanthamine iodide was used instead, a marked increase of the nicotinic response was observed. Submicromolar concentrations of 10-methylgalanthamine iodide resulted in a significant increase in Ca^{2+} influx into the HEK-293-cells. At a concentration of 0.5 µM, potentiation of nicotine-induced Ca^{2+} influx by 54% was measured. Further increase of 10-methylgalanthamine iodide led to inhibition of Ca^{2+} influx due to direct blockade of the nAChR ion channel (cf. Figure 2). For the potentiating effect of 10-methylgalanthamine on the agonist-induced nAChR response an

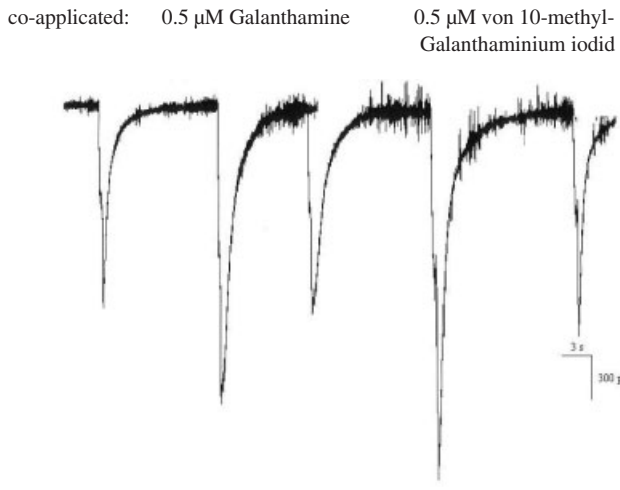

Figure 3. Whole-cell recordings of the effect of galanthamine (1) and 10-methylgalanthamine iodide on nicotine-induced currents through human $\alpha 4\beta 2$ nAChR expressed in HEK-293 cells. Nicotine was applied at 50 µM alone or together with indicated galanthamine derivative. Measurements were done at holding potential −70 mV

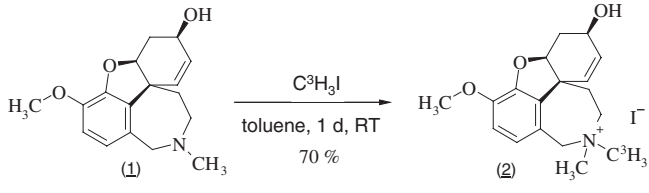

Figure 4. Radioactive labelling of galanthamine (1) using [^{3}H]methyl iodide

EC_{50} value of 270 ± 48 nM and a Hill coefficient of 1.9 ± 0.4 were calculated.

The results of the Ca^{2+} imaging experiments are supported by similar findings obtained by electrophysiological measurements (cf. Figure 3).

The synthesis of 10-[^{3}H]methylgalanthamine iodide was achieved with 10 mCi of [^{3}H]methyl iodide in toluene (10 mCi/ml) at a specific activity of 80 Ci/mmol and 10 mg (34.8 µmol) galanthamine (1) (cf. Figure 4). After a reaction time of 24 h at room temperature, the synthesized 10-[^{3}H]methylgalanthamine iodide was purified according to a procedure by Mulholland *et al.* for the removal of solvent and by-products from

quaternary [^{11}C]methyl-derivatives of amines.[7] After fixation of the tritiated product on a silica gel cartridge, the product was eluted with acetic acid (2%). Obviously, the acetic acid interrupts the strong ionic interactions of the quaternary amine with the material of the cartridge by protonating anionic binding sites such as silanoate or other anionic groups in the SiO_2 matrix thus resulting in the elution of the species. The advantage of this method is that the final product is obtained in a small volume of a solution which can be used for most biological studies either directly or after adjusting the pH. By successive elution with ethanol/toluene and pure ethanol the solvent and the educt were removed. A non-identified polar by-product was separated by using a small volume of 1% acetic acid before elution of the product from the cartridge. After work-up, the pure product could be isolated in radiochemical yields of $>70\%$. By comparison to the corresponding UV-calibration curve, a specific activity of 32 Ci/mmol was determined which is sufficient for the planned binding assays.

Experimental

N.c.a. [^{3}H]methyl iodide was purchased from American Radiolabelled Chemicals and the Silica-Sep-Pak®-Plus cartridge from Waters. Galanthamine hydrobromide (1) was supplied from the Institute of Physiological Chemistry and Pathological Biochemistry of the University of Mainz. The analysis of the eluted fractions was performed with radio-HPLC. To the eluent Omni-Szintisol® by Merck, a cocktail for scintillation measurements was added [eluent/scintillator 1/2 (v/v)] prior to the radio-detection. The HPLC system comprised the following components: UV detector Thermo Separation Products LDC spectromonitor 3000; radio detector Berthold Pseudo-LB 506 C-1 with a Z-200-4 cell; scintillation-adding pump Berthold LB 5035-3; HPLC pump by Sykam S1121; injection valve Rheodyne 7125. The eluent was methanol/0.05 M Na_2HPO_4 [60/40 (v/v)] at a flow rate of 1 ml/min. UV detection was performed at 285 nm. An analytical column by Merck, LiChrospher 100 RP 18 – 5 EC (250 × 4.6 mm) was used. The methanol used as eluent was especially designed for scintillation measurements, the phosphate buffer was LiChropur®-grade and both were purchased from Merck.

For analysing the radiochemical yields, well-defined volumes of the different eluate fractions were taken with a Hamilton syringe, mixed

with 4 ml liquid scintillator (Aquasafe 300 by Zinser Analytic) and measured in a Beckman Coulter LS 6500 scintillation counter.

NMR spectra were recorded on a Bruker 400-MHz-FT-NMR spectrometer DRX 400. The chemical shifts are quoted in δ (ppm), coupling constants in Hz. IR spectra were recorded on a Mattson-FT-IR spectrometer Galaxy 2030 with KBr pills. Other instruments used: CHNS-Analysator Vario EL II by Elementar Analysensysteme for elemental analyses; Finnigan MAT 90 by Thermo Electron for mass spectrometry; Perkin–Elmer Lambda 20 for UV/Vis spectrometry and Perkin–Elmer Polarimeter 241 for determination of optical rotations. Uncorrected melting points were determined with a Electrothermal IA Series 9100.

Synthesis of 10-methylgalanthamine iodide

Galanthamine (1) (0.72 g/2.51 mmol) was dissolved in 120 ml dry diethylether. Two hundred and thirty five microlitres (3.78 mmol, 1.51 eq.) of methyl iodide was added and the mixture stirred under protection from light in an argon atmosphere for 15 h at room temperature. The white precipitate formed was filtered off, washed with diethylether and dried under vacuum (10^{-3} mbar). Recrystallization from hot water and methanol yielded 0.88 g of the desired product (2.05 mmol, 82%) as fine, colourless needles.

^{1}H-NMR-spectrum (in D_2O at 298 K, suppression of water signal by CW-presaturation): 2.04 (1 H; br s; H12′); 2.12 (1 H; ddd; 16,4; 5,3; 3,1; H4′); 2.32 (1 H; br s; H12″); 2.46 (1 H; dt; 16,4; 1,5; H4″); 2.87 (3 H; s; $|N^+$-CH_3); 3.31 (3 H; s; N^+-CH_3); 3.58 (1 H; m; H11′); 3.81 (3 H; s; -OCH_3); 4.04 (1 H; m; H11″); 4.24 (1 H; br t; 4,9; H3); 4.30 (1 H; br d; 13,9; H9′); 5.99 (1 H; dd; 9,8; 4,9; H2); 6.12 (1 H; br s; H1); 6.83 (1 H; br d; 8,3; H8); 6.91 (1 H; d; 8,3; H7). The signals for H4a and H9″, which should lie between 4.5 and 5.0 ppm, are missing as expected from the CW-presaturation; in NMR experiments at higher temperatures these signals could be detected by corresponding downfield shifts mass spectrometry (FD) m/z (% rel. int.): 287.7 (6.5%, galanthamine cation); 303.7 (100%, 10-methylgalanthamine); 304.7 (18.3%, isotope peak) the galanthamine elemental analysis: found: C 47.95%; H 5.38%; N 3,28% (calculated for $C_{18}H_{24}NO_3I \cdot H_2O$: C 48.33%; H 5.86%; N 3.13%).

IR (KBr) [ν (intensity)]: 3555 (s); 3500–3100 (br m); 3010 (w); 2980 (w); 2935 (m); 2900 (w); 1625 (m); 1590 (m); 1480 (s); 1435 (s); 1375 (m);

1280 (s); 1235 (m); 1215 (m); 1195 (m); 1175 (s); 1065 (s); 1030 (m); 1005 (s); 960 (s); 935 (m); 885 (m); 870 (m); 820 (m); 805 (s); 780 (m); 665 (w); 600 (w).

UV (100% EtOH [Uvasol®]): $\lambda_{max} = 288.41$ nm,
optical rotation: $[\alpha]_D^{25} = -98.7°(c = 0,55$ [g/100 ml]; $H_2O)$,
melting point (uncorrected): 280–283°C (decomposition).

Synthesis of 10-[^{3}H]methylgalanthamine iodide

The synthesis of 10-[^{3}H]methylgalanthamine iodide was achieved by using 10 mCi of [^{3}H]methyl iodide galanthamine (1) (9.9 mg/34.5 μmol) was dissolved in 1 ml of toluene in a 5 ml reaction vessel flooded with argon. One millilitre of [^{3}H]methyl iodide solution cooled to −78°C was subsequently transferred via a syringe to the reaction vessel. The reaction mixture was stirred for 24 h and protected from light. After dilution with 2 ml ethanol the product was loaded onto a silica cartridge containing 690 mg of silica gel, which had been conditioned with 20 ml of an ethanol/toluene mixture [50/50 (v/v)]. The elution of the educt, a polar by-product presumably formed as a consequence of radiolysis and the desired product was achieved by successive elution with 20 ml ethanol/toluene [50/50 (v/v)], 5 ml ethanol, 6 ml 1% acetic acid and finally several 5 ml fractions of 2% acetic acid. After passing of each eluent the cartridge was dried with helium. Each fraction was analysed by HPLC and its radioactivity was measured.

Calcium imaging

With using membrane-permeable fura-2/AM indicator calcium-imaging measurements were performed to evaluate the effect of the galanthamine derivatives on nicotine-induced Ca^{2+} influx into HEK-293 cells transfected with $\alpha 4\beta 2$ subtype of nAChR. Cells grown on fibronectin-coated coverslips were loaded for 1 h at room temperature with 4 μM Fura-2/AM (Molecular Probes, USA) and 2 mM Ca^{2+} dissolved in HBSS (10 mM HEPES, 5 mM glucose, 1.3 mM Na_2HPO_4, 4 mM $NaHCO_3$, 137 mM NaCl, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.4 mM KH_2PO_4, 5.4 mM KCl, pH 7.4). After loading, the ester groups of fura-2/AM were immediately cleaved to yield a free, membrane-inpermeable fura-2 chelator. Measurements were performed in 2 mM

Ca^{2+}-containing HBBS, pH 7.4 using an imaging system consisting of an Axiolab 100 microscope (Zeiss, Göttingen, Germany), equipped with an XBO 75W xenon lamp (Osram, München, Germany), a TE-1400 CCD camera (Visitron, Puchheim, Germany), a Ludl MAC 2000 controller (Ludl Electronic Products, Hawthorne, USA) and a Physick LVPS focus device (Visitron, Puchheim, Germany). 10-Methylgalanthamine iodide at a concentration range of 0–25 μM, dissolved in 2 mM Ca^{2+}-con-taining HBSS, was added to the cells together with 50 μM nicotine. Under control condition, 50 μM nicotine was applied alone. The emission fluorescence intensity at 510 nm was measured at two excitation wavelengths of 340 and 380 nm in a 5-s interval. Data analysis was done using a software from Hamamatsu and corresponding EC_{50}s and Hill coefficients were calculated with Microcal Origin 5.0 software.

Electrophysiology

Whole-cell current recordings were performed using an LM-EPC-7 patch-clamp system (List, Darmstadt, Germany) on nAChR-expressing HEK-293 cells cultured 3 days on fibronectin-coated cover slips. The bathing solution was composed of (mM): NaCl 145; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; D-glucose 10; HEPES 10 (pH 7.3; 300 mOsm), and the internal pipette solution contained (mM): CsCl 140 (equilibrated with CsOH); EGTA 11; HEPES 10; $MgCl_2$ 1, (pH 7.3; 300 mOsm). The patch microelectrodes were made from borosilicate glass (external diameter 1.6 mm), and the pipette resistance was measured as 5–7 MΩ when filled with the internal solution. After formation of a high-resistance seal with the cell membrane, capacitance transients were minimized using the C-Fast facility of the system. No additional capacitance and serial resistance compensation was applied. All experiments were performed at room temperature at a holding potential of −70 mV. Whole-cell currents were induced by fast application of 10-methylgalanthamine iodide, dissolved in external solution, using a U-shaped tube positioned near the investigated cell, at a flow rate of 0.5–1.0 ml/min. To prevent accumulation of the test compounds in the bath, the cells were superfused with the bathing solution at the same flow rate. In most experiments, in order to inhibit intrinsic muscarinic responses, 1 μM atropine was included in the bathing solutions and in the solutions applied via the U-tube. Signals were filtered at 3.15 kHz (Bessel),

digitized to 10 kHz and analysed on a PC using the pClamp software package version 6.03 (Axon Instruments, Foster City, USA).

Conclusion

We have synthesized 10-[^{3}H]methylgalanthamine iodide in an overall radiochemical yield of $>70\%$ and a specific activity of 32 Ci/mmol in order to investigate the mechanism of interaction of galanthamine with nicotinic acetylcholine receptors. In calcium imaging, the non-radioactive standard compound 10-methylgalanthamine (2) showed a distinct increase of nicotinic response whereas for the original galanthamine, performance was not applicable due to its tendency to deposit in cell membranes. The EC_{50} value of 270 nM and a Hill coefficient of 1.9 for (2) were calculated from the potentiating effect. Additionally, electrophysiological measurements showed a significant potentiation of the nicotinic effect for (2) compared to galanthamine itself. Therefore, compound (2) seems to be a valuable new candidate for investigating the mechanism of interaction of galanthamine derivatives with nicotinic acetylcholine system.

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