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Synthesis and pharmacological effects of new, *N*-substituted soft anticholinergics based on glycopyrrolate

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Abstract

To reduce the possibility of systemic side-effects in locally administered anticholinergics, two new *N*-substituted glycopyrrolate analogues designed using soft drug design approaches have been synthesized and evaluated in vitro and in vivo. Because stereospecificity is known to be important at muscarinic receptors, the new compounds SGM and SGE also have been prepared as their pure 2*R* isomers, 2*R*-SGM and 2*R*-SGE, by starting from optically pure (−)-cyclopentylmandelic acid, and the corresponding isomers were indeed found to be more active. The new soft glycopyrrolates were chemically more stable under acidic conditions, and the ethyl esters SGE were more stable than the methyl esters SGM. The new compounds were also found to be quite susceptible to extrahepatic metabolism, having half-lives of 20–30 min in rat plasma (in vitro), consistent with their soft nature. Binding studies at human muscarinic receptors (M_1–M_4) and guinea-pig ileum assays found 2*R*-SGM and 2*R*-SGE to have potencies somewhat less than, but close to, those of glycopyrrolate and *N*-methylscopolamine. They caused pupil dilation in rabbit eyes, but their mydriatic effects lasted for considerably less time than that of glycopyrrolate, and they did not induce dilation of the pupil in the contralateral, water-treated eyes, indicating that, in agreement with their soft nature, they are locally active, but safe and with a low potential to cause systemic side-effects.

Introduction

Muscarinic receptor antagonists are frequently used therapeutic agents that inhibit the effects of acetylcholine by blocking its binding to muscarinic cholinergic receptors at neuroeffector sites on smooth muscle, cardiac muscle and gland cells as well as in peripherial ganglia and the central nervous system (CNS) (Brown & Taylor 1996). However, their side-effects, which can include dry mouth, photophobia, blurred vision, urinary hesitancy and retention, decreased sweating, drowsiness, dizziness, restlessness, irritability, disorientation, hallucinations, tachycardia and cardiac arrhythmias, nausea, constipation, and severe allergic reactions (Ali-Melkillä et al 1993; Brown & Taylor 1996), often limit their clinical use and even topical anticholinergics can cause the same unwanted side-effects (Osterholm & Camoriano 1982; Birkhimer et al 1984; Hamborg-Petersen et al 1984). Glycopyrrolate is one of the quaternary ammonium anticholinergics, which have reduced CNS-related side-effects as they cannot cross the blood–brain barrier; however, because glycopyrrolate is eliminated mainly as unchanged drug or active metabolite in the urine, its administration is problematic in young or elderly patients and especially in uraemic patients (Franko et al 1970, 1971; Mirakhur & Dundee 1983; Ali-Melkillä et al 1993; Kirvela et al 1993). To increase the therapeutic index of anticholinergics, we have applied the soft drug approach (Bodor 1984; Bodor & Buchwald 2000) in a number of different designs starting from various lead compounds (Hammer et al 1988, 1991; Kumar et al 1993; Brouillette et al 1996; Kumar & Bodor 1996), and three soft glycopyrrolate analogues with moderate pharmacological potency have also been previously reported (Ji et al 2000, 2002). Here, the design, synthesis and pharmacological properties of two new *N*-substituted soft glycopyrrolate analogues are reported together with the preparation and testing of their pure 2*R* isomers. These novel muscarinic antagonists, just as all other soft drugs, are

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expected to elicit their intended pharmacological effect at the site of application, but to be quickly metabolized into their design-in, inactive metabolite upon entering the systemic circulation and rapidly eliminated from the body, which results in reduced systemic side-effects and increased therapeutic index. In addition to the synthesis, pH profiles, rat blood and plasma in-vitro stabilities, receptor binding affinities (M_1–M_4 pK_i), guinea-pig ileum assay activities (pA_2), and in-vivo mydriatic activities in rabbits are also reported.

Materials and Methods

Materials

Glycopyrrolate (glycopyrronium bromide) was kindly provided by Boehringer Ingelheim Chemicals, Inc. Carbamylcholine bromide (carbachol), atropine methylbromide (atropine MeBr) and scopolamine methylbromide (scopolamine MeBr) were obtained from Sigma Chemical Co. (St Louis, MO). *N*-[^{3}H]-Methyl-scopolamine (NMS) was obtained from Amersham Biosciences UK Limited (Buckinghamshire, UK). Cloned human muscarinic receptor subtypes M_1–M_4 were obtained from Applied Cell Science Inc. (Rockville, MD, USA). Scintiverse BD was from Fisher Scientific Co. (Pittsburgh, PA, USA).

Chemicals used for synthesis were reagent or HPLC grade, and were obtained from Aldrich (Milwaukee, WI) and Fisher Scientific Co. Melting points were taken on Fisher–Johns melting apparatus. NMR spectra were recorded on a Bruker Advance 500 MHz NMR spectrometer and are reported in ppm relative to TMS. Elemental analyses were performed by Atlantic Microlab Inc (Atlanta, GA).

Synthesis

*Racemic cyclopentylmandelic acid (**1**)*
Cyclopentylmagnesium bromide ether solution (100 mL, 2 M; 0.2 mol) was added drop-wise to benzoylformic acid (15 g, 0.1 mol) in 330 mL of anhydrous ethyl ether at 0°C. The mixture was stirred at 0°C for 30 min and at room temperature for 24 h. The reaction mixture was treated with 1 N HCl, and the aqueous solution was extracted with ether. The combined ether solution was treated with K_2CO_3 solution. The potassium carbonate solution was acidified with HCl and extracted with ether twice. The ether solution was dried with anhydrous sodium sulfate and evaporated to give a crude product. The crude product was washed with water to get pure racemic cyclopentylmandelic acid **1** (8.0 g, 36.4%). Needle-like crystals, m.p.: 153–154°C. ^{1}H NMR ($CDCl_3$, 500 MHz): 1.28–1.39, 1.42–1.50, 1.51–1.61, 1.63–1.72 (8H, m, $(CH_2)_4$), 2.93 (1H, p, C**H**C(OH)), 7.26–7.30, 7.33–7.36, 7.65–7.67(5H, m, Ph) ppm.

*Methyl cyclopentylmandelate (**2**)*
To a mixture of racemic cyclopentylmandelic acid *R*/*S*(±)**1** (4.47 g, 20 mmol) and potassium carbonate (7.01 g, 50 mmol) in DMF (50 mL), methyl iodide (8.64 g, 60 mmol) was added at room temperature. The mixture was stirred at room temperature for 2 h and then poured into water and extracted with hexanes three times. Evaporation of the dried hexane extract gave a crude product. Flash chromatography of the crude product on silica gel with 1.5:1 hexanes:methylene chloride gave the pure product **2** (3.02 g, 64%). ^{1}H NMR ($CDCl_3$, 300 MHz): 1.32–1.37, 1.43–1.69 (8H, m, $(CH_2)_4$), 2.90 (1H, p, C**H**C(OH)), 3.74 (1H, s, OH), 3.77 (3H, s, CH_3), 7.25–7.37, 7.63–7.65 (5H, m, Ph) ppm.

N-*Methyl-3-pyrrolidinyl cyclopentylmandelate (**4**)*
A solution of **2** (2.20 g, 9.4 mmol) and *N*-methyl-3-pyrrolidinol (**3**, 1.30 g, 13 mmol) in 40 mL of *n*-heptane was heated until 20 mL of heptane had been distilled. About 0.003 g of sodium was added, and the solution was stirred and heated for 2 h as the distillation was continued. More heptane was added at such a rate as to keep the reaction volume constant. Additional sodium was added at the end of 1 h. The solution was then cooled and extracted with 3 N HCl. The acid extract was made alkaline with concentrated NaOH and extracted three times with ether. Removal of the dried ether solution gave a crude oil. Flash chromatography of the crude product on silica gel with 8:1 EtOAc:EtOH gave pure product **4** (2.053 g, 72%). Analysis for $C_{18}H_{25}NO_3$: calcd C, 71.26; H, 8.31; N, 4.62; found: C, 71.55; H, 8.44; N, 4.68. ^{1}H NMR($CDCl_3$, 500 MHz): 1.27–1.35, 1.40–1.47, 1.54–1.60, 1.75–1.90 (8H, m, $(CH_2)_4$), 2.12–2.30, 2.52–2.57, 2.64–2.81 (6H, m $CH_2NCH_2CH_2$), 2.33, 2.36 (3H, 2s, NCH_3), 2.93 ((1H, p, C**H**C(OH)), 3.83 (1H, bs, OH), 5.23 (1H, m, CO_2CH), 7.23–7.36, 7.64–7.67 (5H, m, Ph) ppm.

Cyclopentylphenylhydroxyacetoxy-1-methyl-1-methoxycarbonylpyrrolidinium bromide, SGM
To compound **4** (0.8235 g, 2.71 mmol) in 30 mL of dry acetonitrile, methyl bromoacetate (1.08 g, 7.06 mmol) was added at room temperature. The mixture was stirred for 2 h. Evaporation of acetonitrile gave a crude product. The crude product was dissolved in a small volume of methylene chloride and then poured into 100 mL of dry ethyl ether to precipitate. This procedure was repeated three times to obtain pure product SGM (0.9912 g, 80%). White powder, m.p.: 192–194°C. Analysis for $C_{21}H_{30}BrNO_5$: calcd C, 55.27; H, 6.63; N, 3.07; found C, 55.11; H, 6.59; N, 3.03. ^{1}H NMR ($CDCl_3$, 500 MHz): 1.23–1.29, 1.31–1.37, 1.41–1.47, 1.53–1.67 (8H, m, $(CH_2)_4$), 2.18–2.23, 2.73–2.80, 4.04–4.16, 4.21–4.25 (6H, m, CH_2 NCH_2CH_2), 2.85 (1H, p, C**H**C(OH)), 3.57 (3H, s, NCH_3), 3.80 (3H, s, CO_2CH_3), 4.66, 4.85 (2H, 2dd, CH_2 CO_2), 5.27 (1H, s, OH), 5.52 (1H, m, CO_2CH), 7.25–7.28, 7.32–7.35, 7.57–7.59 (5H, m, Ph) ppm.

Cyclopentylphenylhydroxyacetoxy-1-methyl-1-ethoxycarbonylpyrrolidinium bromide, SGE
To compound **4** (0.369 g, 1.22 mmol) in 10 mL of dry acetonitrile, ethyl bromoacetate (0.377 g, 2.25 mmol) was added at room temperature. The mixture was stirred for

2 h. Evaporation of acetonitrile gave a crude product. The crude product was dissolved in a small volume of ethylene chloride and then poured into 50 mL of dry ethyl ether to precipitate. This procedure was repeated three times to obtain pure product SGE (0.45 g, 79%). White powder, m.p.: 192–194°C. Analysis for $C_{22}H_{32}BrNO_5$: calcd C, 56.17; H, 6.86; N, 2.98; found: C, 56.14; H, 6.89; N, 2.94. ^{1}H NMR ($CDCl_3$, 500 MHz): 1.35 (3H, t, C**H**$_3$CH$_2$), 1.26–1.33, 1.42–1.47, 1.55–1.67 (8H, m, $(CH_2)_4$), 2.14–2.21, 2.73–2.79, 4.12–4.17, 4.22–4.29 (6H, m, CH_2 NCH_2CH_2), 2.86 (1H, p, C**H**C(OH)), 3.62 (3H, s, NCH_3), 4.25 (2H, q, CH_3C**H**$_2$), 4.67, 4.83 (2H, dd, CH_2 CO_2), 4.91 (1H, s, OH), 5.53 (1H, m, CO_2CH), 7.25–7.27, 7.32–7.34, 7.57–7.59 (5H, m, Ph) ppm.

*Resolution of racemic cyclopentylmandelic acid (**1**)*
(−)-Strychnine (6.10 g) in 50 mL of methanol (suspension) was added to racemic cyclopentylmandelic acid **1** (3.96 g) in methanol (20 mL) at room temperature. The reaction solution was left to stand overnight. The crystal was filtered and crystallized again with hot methanol. The second crop of crystals was collected by filtration and treated with sodium hydroxide solution. The basic solution was extracted with methylene chloride twice (methylene chloride solution discarded), and then acidified with hydrochloric acid to recover the resolved cyclopentylmandelic acid. To this resolved acid (20.6 mg in 0.1 mL of ethyl acetate), 13 μL of (+)-α-phenylethylamine was added. The precipitate formed was washed with hexane three times and dried under vacuum. The precipitate was identified by NMR as optically pure cyclopentylmandelic acid, *R*(−)**1** (1.49 g, 37.6%), m.p.: 121–122°C. $[\alpha]^{25^\circ}{}_D = -22.5^\circ$ ($c = 1$ g 100 mL^{-1}, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz): 1.28–1.39, 1.42–1.50, 1.51–1.61, 1.64–1.73 (8H, m, $(CH_2)_4$), 2.93 (1H, p, C**H**C(OH)), 7.25–7.28, 7.32–7.35, 7.64–7.65 (5H, m, Ph) ppm.

*Methyl (−)-cyclopentylmandelate, R(−)**2***
To a mixture of (−)-cyclopentylmandelic acid, *R*(−)**1**, (1.83 g, 8.3 mmol) and potassium carbonate (2.87 g, 21 mmol) in DMF (21 mL), methyl iodide (3.53 g, 25 mmol) was added at room temperature. The mixture was stirred at room temperature for 2 h, and then poured into water and extracted with hexanes three times. Evaporation of the dried hexanes extract gave a crude product. Flash chromatography of the crude product on silica gel with 1.5:1 hexanes:methylene chloride gave pure product *R*(−)**2** (1.95 g, 100%). Analysis for $C_{18}H_{18}O_3$: calcd C, 71.77; H, 7.74; found C, 71.88; H, 7.80. ^{1}H NMR ($CDCl_3$, 500 MHz): 1.32–1.36, 1.43–1.61 (8H, m, $(CH_2)_4$), 2.90 (1H, p, C**H**C(OH)), 3.71 (1H, s, OH), 3.79 (3H, s, CH_3), 7.25–7.28, 7.31–7.35, 7.63–7.65 (5H, m, Ph) ppm.

N-*Methyl-3-pyrrolidinyl (−)-cyclopentylmandelate, 2R-**4***
A solution of *R*(−)**2** (1.85 g, 7.9 mmol) and *N*-methyl-3-pyrrolidinol (**3**, 1.05 g, 10.4 mmol) in 40 mL of *n*-heptane was heated until 20 mL of heptane had distilled. Approximately 0.003 g of sodium was added and the solution was stirred and heated for 2 h as the distillation was continued. More heptane was added at such a rate as to keep the reaction volume constant. Additional sodium was added at the end of 1 h. The solution was then cooled and extracted with 3 N HCl. The acid extract was made alkaline with concentrated NaOH and extracted three times with ether. Removal of dried ether solution gave a crude oil. Flash chromatography of the crude product on silica gel with 8:1 EtOAc:EtOH gave 2*R*-**4** as a mixture of two diastereoisomers in an NMR-estimated ratio of 1:1 (1.68 g, 70%). Analysis for $C_{18}H_{25}NO_3 \cdot 0.2H_2O$: calcd C, 70.42; H, 8.34; N, 4.5; found C, 70.60; H, 8.26; N, 4.63. ^{1}H NMR ($CDCl_3$, 500 MHz): 1.28–1.37, 1.40–1.47, 1.51–1.70, 1.73–1.80, 1.83–1.90 (8H, m, $(CH_2)_4$), 2.14–2.21, 2.27–2.35, 2.36–2.42, 2.52–2.55, 2.64–2.81 (6H, m, $CH_2NCH_2CH_2$), 2.33, 2.37 (3H, 2s, NCH_3), 2.93 (1H, p, C**H**C(OH)), 3.78 (1H, bs, OH), 5.22 (1H, m CO_2CH), 7.24–7.27, 7.31–7.35, 7.64–7.66 (5H, m, Ph) ppm.

Cyclopentylphenylhydroxyacetoxy-1-methyl-1-methoxycarbonylpyrrolidinium bromide 2R-SGM
To compound 2*R*-**4** (0.15 g, 0.49 mmol) in 6 mL of dry acetonitrile, methyl bromoacetate (0.194 g, 1.27 mmol) was added at room temperature. The mixture was stirred for 6 h. Evaporation of acetonitrile gave a crude product. The crude product was dissolved in a small volume of methylene chloride and then poured into 50 mL of dry ethyl ether to precipitate. This procedure was repeated three times to obtain product 2*R*-SGM (0.1879 g, 83%), as a mixture of four diastereoisomers in an NMR-estimated ratio of 1:1:2:2. White powder, m.p.: 153–155°C. $[\alpha]^{25^\circ}{}_D = +0.5^\circ$ ($c = 1$ g 100 mL^{-1} $CHCl_3$). Analysis for $C_{21}H_{30}BrNO_5 \cdot 0.2H_2O$: calcd C, 54.86; H, 6.62; N, 3.05; found C, 54.75; H, 6.66; N, 3.01. ^{1}H NMR ($CDCl_3$, 500 MHz): 1.30–1.37, 1.41–1.50, 1.55–1.73 (8H, m, $(CH_2)_4$), 1.93–2.00, 2.12–2.26, 2.75–2.95, 3.00–3.03, 4.30–4.50, 4.57–4.61 (7H, m, C**H**C(OH) and $CH_2NCH_2CH_2$), 3.09, 3.30 (1H, 2s, OH), 3.64, 3.66, 3.84, 3.95, 3.97 (3H, 5s, NCH_3), 3.74, 3.77, 3.79, 3.81 (3H, 4s, CO_2CH_3), 4.78, 4.83; 4.90, 4.97; 5.30, 5.35; 5.37, 5.41 (2H, four groups of 2dd, CH_2CO_2), 5.53 (1H, m, CO_2CH), 7.23–7.29, 7.31–7.38, 7.56–7.60 (5H, m, Ph) ppm.

Cyclopentylphenylhydroxyacetoxy-1-methyl-1-ethoxycarbonylpyrrolidinium bromide, 2R-SGE
To compound 2*R*-**4** (0.22 g, 0.73 mmol) in 10 mL of dry acetonitrile, ethyl bromoacetate (0.21 mL, 0.316 g, 1.89 mmol) was added at room temperature. The mixture was stirred for 22 h. Removal of acetonitrile gave a crude product. The crude product was dissolved in a small volume of ethylene chloride and then poured into 50 mL of dry ethyl ether to precipitate. This procedure was repeated three times to obtain product 2*R*-SGE (0.3085 g, 90%) as a mixture of four diastereoisomers in an NMR-estimated ratio of 1:1:2:2. White powder, m.p.:143–145°C. $[\alpha]^{25^\circ}{}_D = +5.6^\circ$ ($c = 1$ g 100 mL^{-1} $CHCl_3$). Analysis for $C_{22}H_{32}BrNO_5 \cdot 0.3H_2O$: calcd C, 55.53; H, 6.91; N, 2.94; found C, 55.46; H, 6.85; N, 2.97. ^{1}H NMR ($CDCl_3$,

500 MHz): 1.26, 1.28, 1.32, 1.35 (3H, 4t, C**H**$_3$CH$_2$), 1.44–1.50, 1.53–1.63, 1.65–1.70 (8H, m, (CH$_2$)$_4$), 1.93–2.00, 2.04–2.11, 2.18–2.25, 2.76–2.96, 3.01–3.04, 4.09–4.26 (7H, m, C**H**C(OH) and CH$_2$NCH$_2$CH$_2$), 3.06, 3.28 (1H, 2s, OH), 3.66, 3.69, 3.81, 3.82, 3.94, 3.96 (3H, 6s, NCH$_3$), 4.61, 4.69; 4.76, 4.85; 5.17, 5.22; 5.26, 5.30 (2H, four sets of dd, CH$_2$CO$_2$), 4.26–4.52 (2H, m, CH$_3$C**H**$_2$), 5.53 (1H, m, CO$_2$CH), 7.24–7.29, 7.31–7.38, 7.56–7.60 (5H, m, Ph) ppm.

Methods

pH profile

The stabilities of the soft glycopyrrolates in standard phosphate buffers (0.05 M) of various pHs (6.00–8.40) were investigated at 37°C. Aliquots of 4.4 mM of the compounds in water solution were added to the buffer solutions to give a final concentration of 0.44 mM. At appropriate time intervals, samples were taken and analysed by HPLC to monitor the disappearance of the soft analogues and the formation of hydrolysis products. The pseudo first-order rate constant (k, min^{-1}) and half-life ($t_{1/2}$, min) of the disappearance of the compound in the buffer were calculated.

In-vitro studies

The stability of soft glycopyrrolates in biological media in vitro was determined by measuring the pseudo first-order rate constants (k, min^{-1}) and half-lives ($t_{½}$, min) of the disappearance of the compound in rat blood and plasma. Aliquots of 22 mM were added to the biological medium at 37°C to yield a final concentration of 0.7 mM. At appropriate time intervals samples (0.15 mL) were withdrawn and mixed with 0.3 mL of 5% DMSO in acetonitrile solution. The mixtures were centrifuged and the supernatants were analysed by HPLC. Experiments were performed in triplicate.

Analytical method

The HPLC system used for the analysis of the soft analogues and their hydrolysis products was as follows. A Supelcosil LC-8 column (25 cm × 4.6 mm) was used with a mobile phase of acetonitrile (42%) and aqueous solution (58%) containing sodium phosphate (10 mM), acetic acid (0.1%) and triethylamine (0.1%). At a flow rate of 1 mL min^{-1} the retention times were 6.02 min (SGM and +SGM), 7.27 min (SGE and +SGE) and 4.14 min (hydrolysis product), respectively. With an injection volume of 10 μL, the detection limit was 1 μg mL^{-1}.

Receptor binding affinity

Receptor binding studies were performed with NMS in assay buffer (phosphate-buffered saline, PBS, without Ca^{++} or Mg^{++}, pH 7.4) following the protocol obtained from Applied Cell Science Inc. (Rockville, MD, USA). A 10 mM NaF solution was added to the buffer as an esterase inhibitor. The assay mixture (0.2 mL) contained 20 μL diluted membranes (receptor proteins, final concentration: M_1, 38 μg mL^{-1}; M_2, 55 μg mL^{-1}; M_3, 27 μg mL^{-1}; M_4, 84 μg mL^{-1}). The final concentration of NMS for the binding studies was 0.5 nM. Specific binding was defined as the difference in [^{3}H]NMS binding in the absence and presence of 5 μM atropine for M_1 and M_2 or 1 μM atropine for M_3 and M_4. Incubation was carried out at room temperature for 120 min. The assay was terminated by filtration through a Whatman GF/C filter (presoaked with 0.5% polyethyleneimine). The filter was then washed six times with 1 mL ice-cold buffer (50 mM Tris-HCl, pH 7.8, 0.9% NaCl), transferred to vials and 5 mL of Scintiverse was added. Final detection was performed on a Packard 31800 liquid scintillation analyser (Packard Instrument Inc., Downer Grove, IL). Data obtained from the binding experiments were fitted to the %[^{3}H] NMS bound $= 100 - (100x^n/k/(1 + x^n/k))$ equation, to obtain the Hill coefficient n, and then to %[^{3}H] NMS bound $= 100 - (100x^n/IC_{50}/(1 + x^n/IC_{50}))$, to obtain IC_{50}s (x being the concentration of the tested compound). Based on the method of Cheng and Prusoff (Cheng & Prusoff 1973), K_i was derived from the equation $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of the radioligand. IC_{50} represents the concentration of the drug causing 50% inhibition of specific radioligand binding and K_d represents the dissociation constant of the radioligand receptor complex. Experiments were performed in triplicate. Data were analysed by a non-linear least-square curve-fitting procedure using Scientist software (MicroMath Inc., Salt Lake City, UT, USA).

pA_2 values

Male guinea-pigs weighing about 400 g were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN) and used after overnight fasting. Animals were sacrificed by decapitation and the ileum (the region of 5 cm upward of the caecum) was isolated and removed. The ileum was cut into 2.5-cm pieces and suspended in an organ bath containing 30 mL of a mixture of Tyrode's solution and 0.1 mM hexamethonium bromide. The organ bath was constantly aerated with oxygen and kept at 37°C. One end of the ileum strip was attached to a fixed support at the bottom of the organ bath and the other end to an isometric force transducer (Model TRN001, Kent Scientific Corp., CT, USA) operated at 2–10 g range. The ileum strip was kept at a 2 g tension and carbachol was used as antagonist. The ileum contracted cumulatively upon the addition of consecutive doses of carbachol (10–20 μL of $2 \times 10^{-4} - 2 \times 10^{-3}$ M in water solution). Contractions were recorded on a physiograph (Kipp & Zonen Flarbed Recorder, Holland). After the maximum response was achieved, the ileum was washed three times and a fresh Tyrode's solution containing an appropriate concentration of the antagonist (SGM, SGE, 2*R*-SGM, 2*R*-SGE, glycopyrrolate, or scopolamine) was replaced. An equilibration time of 10 min was allowed for the antagonists before the addition of carbachol. Four to six trials were performed for each antagonist.

Pharmacological activities of soft glycopyrrolates

The mydriatic effects of the soft analogues in rabbit eyes have been compared with those of glycopyrrolate. Four healthy male New Zealand white rabbits weighing about 3.5 kg were used. To investigate the dose–mydriatic-

response relationships, 100 μL of various concentrations of the compounds (0, 0.5 and 1% for the soft drugs and 0, 0.05, 0.1 and 0.2% for glycopyrrolate) were administered to the eyes to determine the pharmacodynamically equivalent doses, i.e. the lowest doses that induce the maximum pupil dilations. Drug solutions were applied to one eye; only water was applied to the other eye, which served as a control. Experiments were carried out in a light- and temperature-controlled room. At appropriate time intervals, the pupil diameters of both eyes were recorded. The differences in pupil diameters between each time-point and the zero time-point were calculated for both treated and control eyes and reported as mydriatic responses ((treated–control)/control in %). Control eye dilations were monitored to determine whether or not systemic absorption had occurred. For each compound, four trials were conducted. Animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health, USA. Institutional animal care and use committee (IACUC) approval was obtained prior to the initiation of this research and during its execution.

Statistical analysis

Stability, receptor binding and pA_2 activities were compared using both *t*-tests and nonparametric Mann–Whitney U-tests for the compound pairs of interest. Pharmacological activities (maximum response R_{max}% and area under the effect curves AUC^{eff}) were compared using ANOVA followed by Tukey–Kramer multiple comparison tests as a parametric post hoc test (Jones 2002). A significance level of $P < 0.05$ was used in all cases. All statistical analyses were performed using NCSS (Number Cruncher Statistical Systems, Kaysville, UT, USA).

Results and Discussion

Synthesis

The new soft glycopyrrolate analogues, SGM and SGE (cyclopentylphenylhydroxyacetoxy-1-methyl-1-(alkoxy-carbonyl)pyrrolidinium bromide; alkoxy- being methoxy- and ethoxy- for SGM and SGE, respectively), were synthesized as shown in Figure 1 except for the second, resolution step. This involved (i) Grignard reaction of cyclopentylmagnesium bromide with benzoylformic acid in anhydrous ether to give the racemic cyclopentylmandelic acid **1**; (ii) methylation of **1** with methyl iodide and potassium carbonate in DMF at room temperature to yield methyl cyclopentylmandelate **2**; (iii) transesterification (Franko & Lunsford 1960) of **2** with 1-methyl-3-pyrrolidinol (**3**) in heptane to give *N*-methyl-3-pyrrolidinyl cyclopentylmandelate **4**; and (iv) quaternization of **4** with alkyl bromoacetate in acetonitrile to give the final product **5** (SGM and SGE). These are racemic soft glycopyrrolate analogues, and they were characterized by NMR and elemental analysis.

Because stereospecificity is known to be important at muscarinic receptors, improved anticholinergic activity being obtained with the 2*R* configuration of glycopyrrolate-type substances (Barlow et al 1973; Pauling & Datta 1980), these soft drug candidates were also prepared starting with optically pure cyclopentylmandelic acid, *R*(−)**1**. Racemic **1** was resolved by repeated crystallization of the salts produced between acid **1** and (−)-strychnine (Pavia et al 1988; March 1992). Optically pure free acid was recovered by basification of the salts with sodium hydroxide solution followed by acidification with hydrochloric acid. The obtained left rotatory (−22.5°) optically pure *R*(−)**1** was characterized by NMR. Grover and coworkers

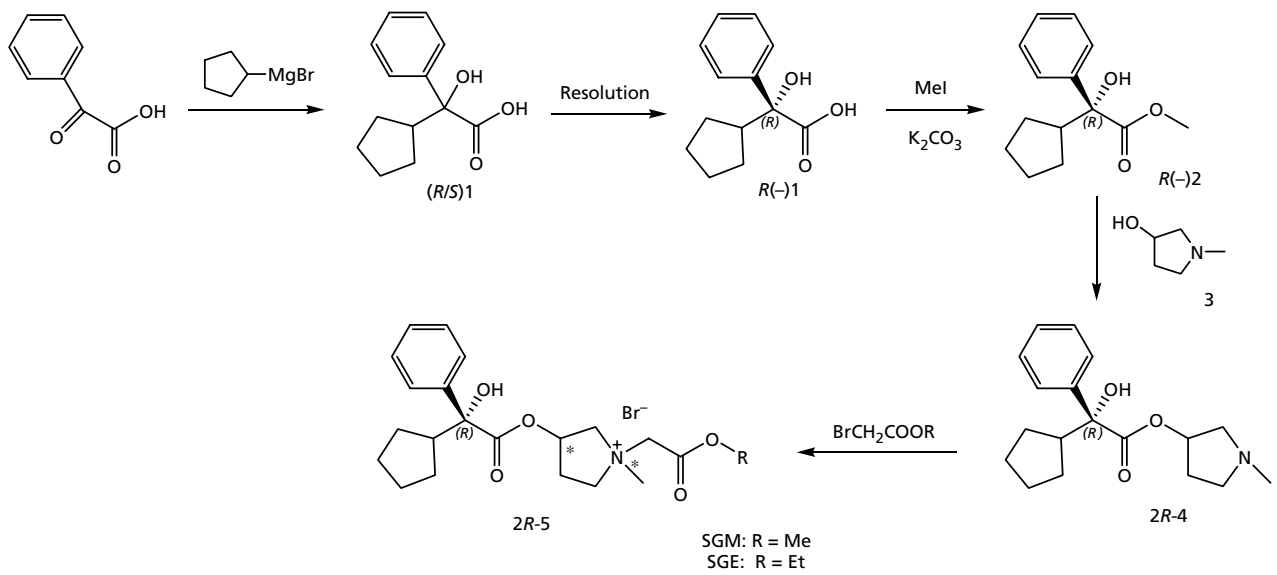

Figure 1 Synthesis of the soft anticholinergics of the present study.

reported the highly stereoselective synthesis of (*S*)-cyclopentyl-phenylglycoxilic acid using (*S*)-mandelic acid (Grover et al 2000) and they found (*S*)-cyclopentyl-phenylglycoxilic acid to have positive optical rotation. Accordingly, *R*(−)**1**, which was found to have an optical rotation of $[\alpha] = -22.5°$, is the *R* form. The NMR (Jacobus & Raban 1969) of the salt formed by the resolved cyclopentylmandelic acid *R*(−)**1** and (+)-α-phenylethylamine gave a single pentaplet for the C**H**C(OH) group, whereas the salt of the unresolved **1** and (+)-α-phenylethylamine gave two pentaplets for C**H**C(OH).

The soft glycopyrrolate analogues 2*R*-SGM and 2*R*-SGE, which have 2*R* configurations, were synthesized from *R*(−)-cyclopentylmandelic acid *R*(−)**1** by the route shown in Figure 1, and they were also characterized by NMR and elemental analysis. The optical rotations of 2*R*-SGM and 2*R*-SGE were + 0.5° and + 5.6°, respectively.

The racemic SGM and SGE had much simpler NMR spectra than their corresponding resolved analogues, 2*R*-SGM and 2*R*-SGE. These molecules have a total of three chiral centres, as shown in Figure 1. In 2*R*-SGM and 2*R*-SGE, one of the chiral centres was resolved, but two others remained; hence, they are both a mixture of four diastereoisomers, complicating their NMR spectra. For example, the $C\mathbf{H}_3CH_2$ methyl group showed only one triplet at 1.35 ppm in SGE, where it is not subject to unequal chemical environments; however, it showed four triplets at 1.26, 1.28, 1.32 and 1.35 ppm, respectively, in 2*R*-SGE, which has one resolved and two unresolved chiral centres and is a mixture of four diastereoisomers (*RRR*, *RSR*, *RRS*, *RSS*). Also, the AB system of SGE's CH_2CO_2 group showed one set of double-doublet signals at 4.67 and 4.83 ppm, but the same system in 2*R*-SGE showed four sets of double-doublet signals at 4.61, 4.69; 4.76, 4.85; 5.17, 5.22 and 5.26, 5.30 ppm.

pH profile

In the pH range of 6.00–8.40 and at 37°C, the chemical hydrolysis of the present soft glycopyrrolate analogues was significantly pH dependent. As shown in Figure 2, these compounds are more stable under acidic conditions, and the ethyl derivatives are more stable than the corresponding methyl derivatives. The half-lives of SGM, 2*R*-SGM, SGE and 2*R*-SGE in aqueous solution at pH 6.0 were 91, 77, 155 and 134 h, respectively. However, at pH 8.4, the corresponding half-lives decreased to 8, 7, 16 and 12 min, respectively. The pH profiles are displayed in Figure 2, and the results indicate a base-catalysed hydrolysis of the compounds with a correlation coefficient of 0.997–0.998. For illustrative purposes, the time profile of the disappearance of 2*R*-SGM and the concurrent formation of the corresponding acid at pH 7.4 is shown in Figure 3.

In-vitro stability

In-vitro stability studies have been performed using rat blood and plasma by measuring the pseudo first-order rate constant (k, min^{-1}) and half-life ($t_{½}$, min) of the disappearance of the parent compounds (Table 1). At 37°C and pH 7.4, the hydrolysis of soft glycopyrrolate analogues was relatively fast in plasma with half-lives of

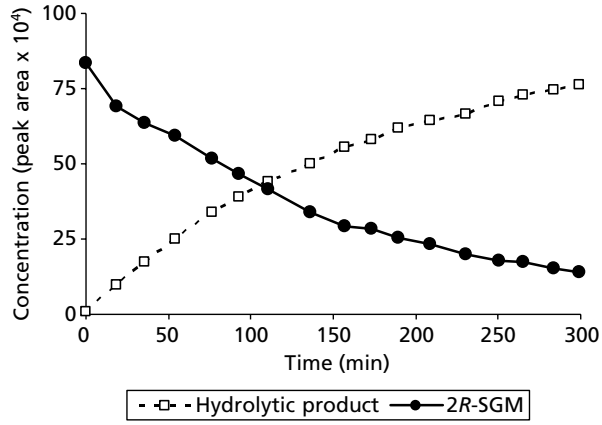

Figure 3 A representative time profile of a chemical hydrolysis with formation of the corresponding acid as the hydrolytic product (2*R*-SGM, pH 7.3, 37 °C).

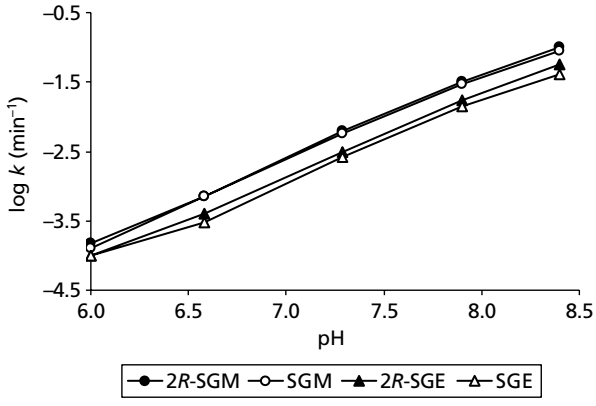

Figure 2 The pH profiles of the present soft anticholinergics.

Table 1 Pseudo first-order rate constant (k, min^{-1}) and half-life ($t_{½}$, min) for the disappearance of soft analogues in rat plasma and blood

Compound	Medium	$k \times 10^{-3}$ (min^{-1})	$t_{½}$ (min)	r^2
SGM	Plasma	36.4 ± 5.0	19.5 ± 2.7	0.998
	Blood	12.3 ± 1.3	57.0 ± 5.8	0.998
SGE	Plasma	15.8 ± 0.2	44.0 ± 0.4	0.997
	Blood	7.2 ± 0.2	96.6 ± 2.9	0.996
2*R*-SGM	Plasma	34.5 ± 3.2	20.0 ± 2.1	0.993
	Blood	12.4 ± 1.4	56.7 ± 6.1	0.997
2*R*-SGE	Plasma	20.9 ± 3.0	33.8 ± 4.8	0.998
	Blood	8.0 ± 0.1	86.4 ± 1.2	0.998

Data represent mean ± s.d. of three experiments.

19.5, 20, 44 and 34 min for SGM, 2*R*-SGM, SGE and 2*R*-SGE, respectively, and significantly slower in blood (57, 57, 97 and 86 min, respectively; $P < 0.05$ for all compounds, *t*-test or nonparametric Mann–Whitney U-test), indicating that blood cell binding is significant enough to slow the hydrolytic degradation of these esters. The ethyl esters were more stable than the methyl derivatives ($P < 0.05$, *t*-test or nonparametric Mann–Whitney U-test).

In-vitro pharmacodynamic evaluation

To evaluate the relative potency of the newly synthesized soft analogues, receptor binding affinities, pK_i and guinea-pig ileum contraction ability, pA_2, were determined.

Receptor binding studies

The receptor binding affinities of soft analogues determined by radioligand binding assays using human cloned muscarinic receptor subtypes, M_1–M_4 are presented in Table 2. The 2*R* isomers 2*R*-SGM and 2*R*-SGE had pK_i values that are in the 8.7–9.5 range; somewhat less than, but close to, those observed for the known highly active antagonists that served as the lead for the present design, *N*-methylscopolamine and glycopyrrolate (9.2–9.9 and 8.7–9.9, respectively). As expected, the racemic forms, SGM and SGE, showed lower receptor binding affinities than their corresponding 2*R* isomers (differences significant at $P < 0.05$ level for M_3, *t*-test or nonparametric Mann–Whitney U-test), confirming that stereospecificity is important at these receptors (Barlow et al 1973; Pauling & Datta 1980).

pA_2 studies

The pA_2 values determined from guinea-pig ileum contraction assays, which represent the negative logarithm of the molar concentration of the antagonist that produces a two-fold shift to the right in an agonist's concentration–response curve, are a classical functional study of anticholinergic affinity (at M_3 muscarinic receptors) (Cheng & Prusoff 1973). For the soft anticholinergics of the present study, the pA_2 values obtained from ileum longitudinal contractions by using carbachol as agonists with the method of van Rossum (van Rossum 1963) (Table 2) were, in general, comparable with the pK_i values obtained in the M_3 receptor binding studies. The 2*R* isomers were again significantly more active than the corresponding racemates, and the most active soft analogue (2*R*-SGE, $pA_2 = 8.55 \pm 0.16$) showed activity similar to glycopyrrolate ($pA_2 = 8.57 \pm 0.12$).

Mydriatic activities of soft analogues

The mydriatic effects of the new soft analogues SGM and SGE were compared to those of glycopyrrolate in rabbits. Mydriatic responses were recorded at appropriate time intervals after the administration of the drugs as percentage changes in pupil size. Maximum response (R_{max}, % change in pupil size at 1 h after administration) and area under the response–time curve (AUC^{eff}) are shown in Table 3. Whereas there are no significant differences among the R_{max} maximum responses among all treatments considered (compounds and concentrations of Table 3), there clearly are significant differences among the AUC^{eff} values ($P < 0.05$, ANOVA followed by Tukey–Kramer multiple comparison test). Glycopyrrolate (0.1%, 0.2%) gave significantly longer-lasting effects (larger AUC^{eff}) than any of the soft analogues. The soft ethyl analogues seem somewhat more potent than corresponding methyl analogues, and the 2*R* isomers seem more potent than the isomeric mixtures. In agreement with soft drug design principles, their duration of action is much shorter than that of the 'hard' glycopyrrolate, as illustrated in Figure 4 for pharmacodynamically equipotent doses. The mydriatic activity of 2*R*-SGM, 2*R*-SGE and glycopyrrolate lasted for 24, <48 and 144 h,

Table 2 Receptor binding affinities and pA_2 values

Compound	Subtypes of cloned muscarinic receptors[a]				pA_2[b]
	M_1	M_2	M_3	M_4	
2*R*-SGM	8.89 ± 0.04	8.87 ± 0.05	9.00 ± 0.06	9.52 ± 0.01	8.31 ± 0.05
	(0.83 ± 0.11)	(1.10 ± 0.11)	(0.83 ± 0.01)	(0.83 ± 0.01)	
SGM	7.91 ± 0.05	7.79 ± 0.11	7.80 ± 0.10	8.29 ± 0.19	7.90 ± 0.13
	(1.02 ± 0.12)	(1.25 ± 0.01)	(1.17 ± 0.18)	(1.12 ± 0.05)	
2*R*-SGE	8.67 ± 0.16	8.84 ± 0.34	8.74 ± 0.02	8.85 ± 0.13	8.55 ± 0.16
	(0.86 ± 0.08)	(0.92 ± 0.01)	(1.09 ± 0.15)	(0.89 ± 0.02)	
SGE	7.51 ± 0.17	7.32 ± 0.07	7.54 ± 0.15	7.94 ± 0.09	7.36 ± 0.34
	(0.91 ± 0.09)	(1.23 ± 0.06)	(1.18 ± 0.08)	(1.18 ± 0.09)	
Glycopyrrolate	9.76 ± 0.05	9.19 ± 0.18	8.73 ± 0.05	9.90 ± 0.08	8.57 ± 0.12
	(1.37 ± 0.20)	(0.99 ± 0.11)	(1.14 ± 0.25)	(1.02 ± 0.01)	
Scopolamine methyl bromide	9.69 ± 0.01	9.18 ± 0.21	9.29 ± 0.12	9.92 ± 0.21	9.16 ± 0.19
	(0.92 ± 0.10)	(1.02 ± 0.02)	(1.07 ± 0.01)	(0.90 ± 0.04)	

[a]Data of the receptor binding experiments represent mean ± s.d. of three experiments. The numbers in parentheses denote Hill slopes. [b]pA_2 values were determined on four to six ileum strips obtained from different animals. Data represent mean ± s.d.

Table 3 Maximum response (R_{max}, percentage change in pupil size at 1 h after administration) and area under the response–time curve (AUC^{eff})

Compound	Concn (%)	R_{max} (%)	$AUC^{eff}_{(0-144\,h)}$
SGM	0.5	45.83 ± 4.81	185 ± 35
	1	59.58 ± 15.72	467 ± 114
SGE	0.5	44.65 ± 13.99	596 ± 274
	1	58.33 ± 12.27	645 ± 409
2*R*-SGM	0.5	52.92 ± 13.41	752 ± 342
	1	57.08 ± 11.66	875 ± 197
2*R*-SGE	0.5	53.96 ± 13.27	1170 ± 308
	1	56.04 ± 11.69	1532 ± 526
Glycopyrrolate	0.05	51.46 ± 7.71	2779 ± 443
	0.1	55.83 ± 6.42	4074 ± 459
	0.2	56.04 ± 10.10	5047 ± 1631

Data indicate mean ± s.d. of four trials.

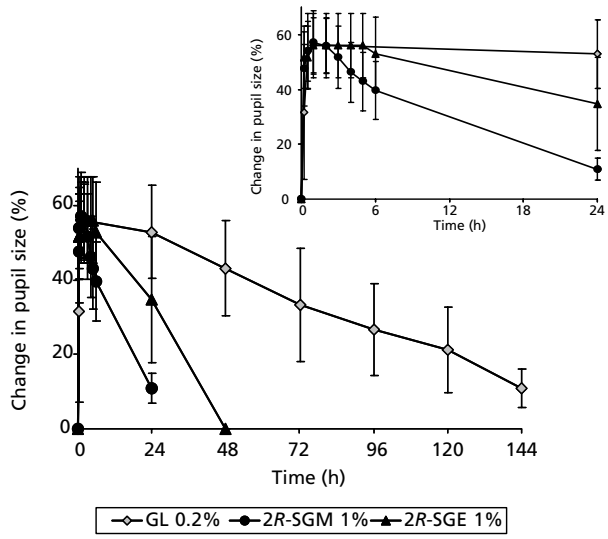

Figure 4 Mydriatic activities of glycopyrrolate (GL) and soft analogues at pharmacodynamically equipotent doses (mean ± s.d. shown, and the smaller inset shows data for the first 24 h only).

respectively, indicating that soft analogues are easily hydrolysed and rapidly eliminated from the body after the desired pharmacological effect is achieved. In agreement with this and unlike other traditional anticholinergics, these soft drugs did not induce dilation of the pupil in the contralateral (water-treated) eye, indicating no or only low systemic side-effects. These compounds are therefore safe, promising short-acting anticholinergics with the possibility of largely reduced unwanted side-effects.

In conclusion, a set of new glycopyrrolate-based soft anticholinergics has been designed, synthesized and tested. They were found to have receptor-binding affinities comparable to those of glycopyrrolate or *N*-methylscopolamine and good, but short-lasting, mydriatic activity with no or minimal systemic effects due to their soft nature, which allows easy, one-step metabolism into a designed-in metabolite after exerting their desired pharmacological activity.

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