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Research Paper

Pharmacokinetic and Pharmacodynamic Evaluations of the Zwitterionic Metabolite of a New Series of *N*-Substituted Soft Anticholinergics

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Purpose. This study was conducted to evaluate the zwitterionic common metabolite of a novel series of *N*-substituted soft analogs of glycopyrrolate both as racemates and as 2*R* isomers.
Methods. Activities were assessed using both *in vitro* (receptor binding assay, guinea pig ileum pA_2 assay) and *in vivo* techniques (rabbit mydriatic response, rat cardiac effects). Pharmacokinetic characterizations in rats were also performed.
Results. The metabolite was highly water-soluble and very stable in buffer solutions as well as in rat biological media. Following i.v. administration in rats, it was very rapidly eliminated, mainly through renal excretion with a half-life of about 10 min. Receptor binding and guinea pig ileum assays indicated this metabolite as more than 1 order of magnitude less active than its parent soft drugs or glycopyrrolate. Moderate M_3/M_2 muscarinic receptor subtype selectivity was observed, further reducing the likelihood of cardiac side effects. The metabolite showed to some extent mydriatic effect and protective effect against carbachol-induced bradycardia, but of much shorter durations than glycopyrrolate; it had, however, no effect on resting heart rate.
Conclusions. *N*-Substituted zwitterionic metabolites retain some, but only considerably reduced activity of their parent quaternary ammonium ester soft anticholinergic drugs, and they are very rapidly eliminated from the systemic circulation. They are suitable for their assigned role within the framework of inactive metabolite-based soft anticholinergic design.

KEY WORDS: glycopyrrolate; guinea pig ileum assay; muscarinic antagonist; mydriatic effect; stereoselectivity.

INTRODUCTION

Muscarinic receptor antagonists inhibit the effects of acetylcholine at muscarinic cholinergic receptors at neuroeffector sites on smooth muscle, cardiac muscle, and gland cells as well as in peripherial ganglia and in the central nervous system (CNS), and they are frequently used to treat various clinical conditions, such as asthma, peptic ulcer, or Parkinson's disease, as mydriatic/cycloplegic agents (1,2), and even as experimental antiperspirants (3–7). However, the use of anticholinergics is often limited by their unwanted and prolonged systemic side effects, even after ophthalmic administrations (8–15). To develop anticholinergics with maximal local therapeutic benefits and minimal systemic side effects, the soft drug design approach has been used starting from various lead compounds ever since the first related publication in 1980 (16–18). The soft anticholinergics obtained from these designs are structural analogs of known anticholinergics and, just as all other soft drugs, are expected to elicit their intended pharmacological effect at the site of application, but to be quickly metabolized into their designed-in inactive metabolite upon entering the systemic circulation and rapidly eliminated from the body, resulting in reduced systemic side effects and increased therapeutic indices (16–26).

Glycopyrrolate, which served as the lead for the soft anticholinergic designs related to the present study, is a quaternary ammonium compound with hindered passage across lipid membranes (including the blood–brain barrier) and, hence, with reduced CNS-related side effects compared to atropine or scopolamine. However, its peripheral side effects, such as dry mouth, dry skin, tachycardia, visual disturbances, and difficulty in urination, can last up to 8 h after an intramuscular dose of 8 $\mu g\ kg^{-1}$. Also, because glycopyrrolate is mainly eliminated as unchanged drug or active metabolite in the urine, it can induce prolonged drug effects in the elderly uremic patients (27,28). The design, synthesis, and *in vitro*/*in vivo* testing of three soft analogs of glycopyrrolate that were found to have promising local anticholinergic activity, but minimal or zero systemic side effects have been reported previously (29,30).

Anticholinergics with a quaternary ammonium head in general tend to show somewhat better activity than their corresponding neutral forms (e.g., methylatropine *vs.* atropine, or *N*-methylscopolamine *vs.* scopolamine), but the neutral compounds are still highly active. The acidic metabolites of

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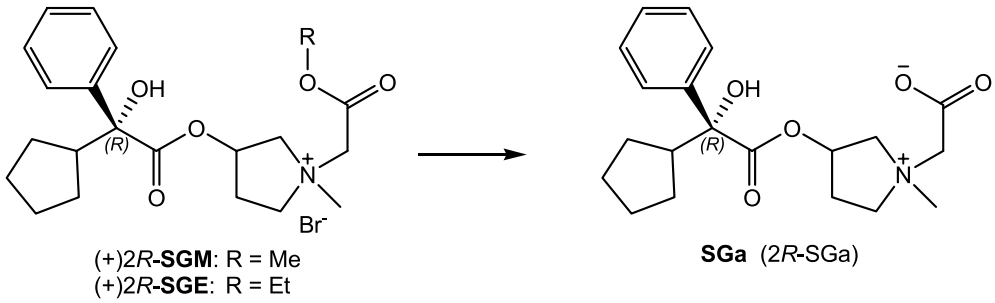

Fig. 1. Structures of the *N*-substituted soft glycopyrrolate analogs and their corresponding zwitterionic metabolite SGa.

previously developed inactive metabolite-based soft anticholinergics were shown to be essentially inactive, as required by the principle of one-step deactivation of soft drug design (31–33). However, our more recently developed series of *N*-substituted soft anticholinergics (29,30,34,35) have a zwitterionic metabolite such as SGa (Fig. 1), in which the positive quaternary nitrogen and the negative acid moiety formed by hydrolysis are spatially very close, and hence the overall electron distribution is somewhat similar to that of the neutral compound, which is active (Fig. 2). Therefore, because this metabolite might still retain some activity (most likely more than the previous acidic metabolites with a differently placed acid moiety), we undertook a detailed investigation of its pharmacokinetics and pharmacodynamics (PK/PD) to ensure that the corresponding *N*-substituted soft anticholinergics can still be considered as undergoing a facile, essentially one-step metabolic deactivation as required by the principles of soft drug design. Because stereo-

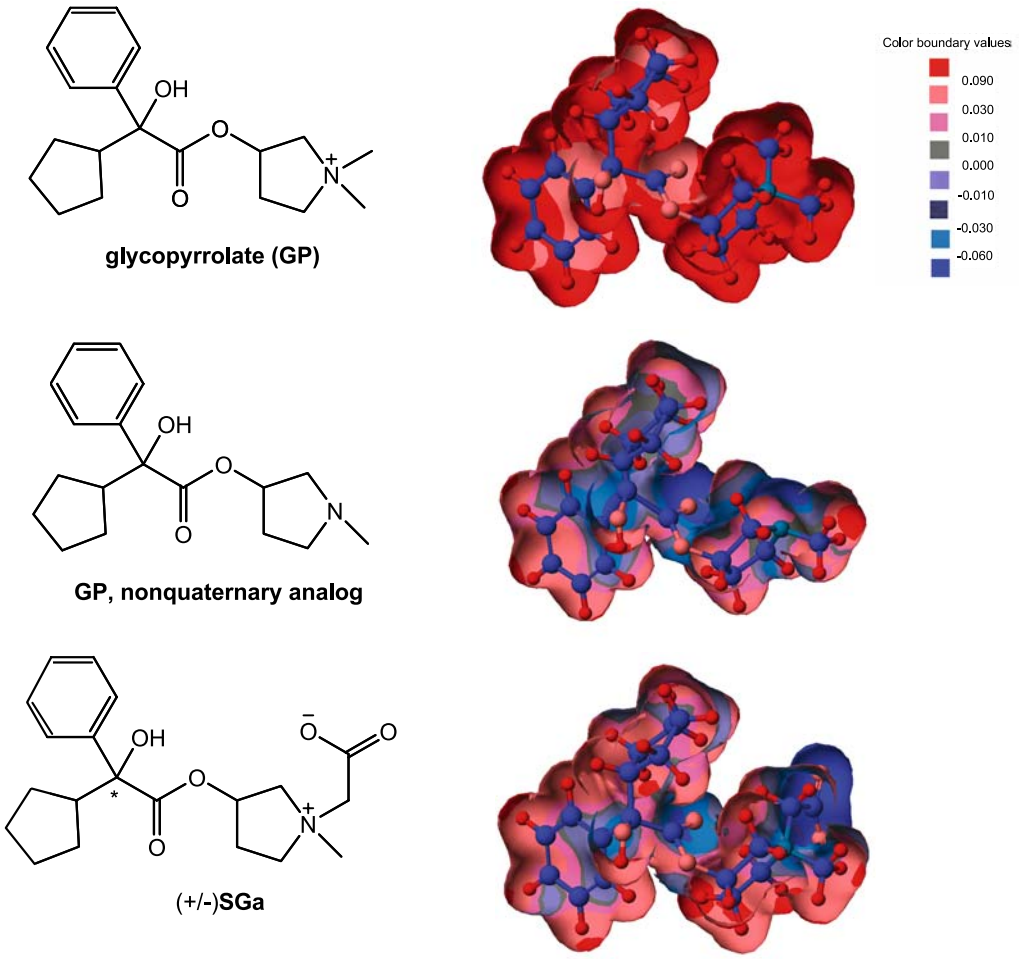

Fig. 2. Structures and electron isodensity surfaces for glycopyrrolate (GP), its nonquaternary analog, and the zwitterionic metabolite (±)SGa of *N*-substituted soft glycopyrrolate analogs. Electron-isodensity surfaces (0.01 electron $Å^{-3} \sim 0.0015$ electron $bohr^{-3}$) of the fully AM1-optimized structures are colored according to the electrostatic potential. The color code changes gradually from blue, which corresponds to the more negative regions, to red, which corresponds to the more positive regions along the surface.

specificity is known to affect pharmacological activity at muscarinic receptors (36–40), in addition to the racemic metabolite (±)SGa, 3-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-methyl-1-carboxymethylpyrrolidinium inner salt, the corresponding 2*R* isomer 2*R*-SGa (henceforth denoted simply as SGa) was also prepared and examined as for glycopyrrolate-type structures the 2*R* isomers are known to be more active.

Receptor binding affinity is a major determinant of drug activity. For muscarinic receptors, five subtypes, M_1–M_5, have been found and cloned from human tissue (41–43), and there is sufficient correlation among these molecular subtypes and pharmacological subtypes to warrant the use of a unified M_1–M_5 notation (44). Subtype selectivity (e.g., M_3/M_2) could be useful in eliminating many potential side effects, but most currently used anticholinergics show no subtype selectivity; a few newer ones that show muscarinic receptor subtype selectivity are being pursued for development (45). For soft anticholinergics, such subtype selectivity could also further enhance their therapeutic advantage by further decreasing their side effects, and slight subtype selectivity has already been achieved with some *N*-substituted soft anticholinergics (35).

In the present study, chemical and biological stabilities have been evaluated *in vitro* in aqueous solutions and in rat blood, plasma, and lung and liver homogenates. *In vitro* anticholinergic activities were characterized through M_1–M_4 receptor binding affinities (pK_i), and through guinea pig ileum assay pA_2 values. *In vivo* pharmacological activities were evaluated through mydriatic effects in rabbits and cardiac effects in rats. Pharmacokinetics, after i.v. administration in rats, has also been evaluated.

MATERIALS AND METHODS

Materials

Glycopyrrolate (glycopyrronium bromide) was kindly provided by Boehringer Ingelheim Chemicals (Petersburg, VA, USA). Carbamylcholine bromide (carbachol), atropine methylbromide (atropine MeBr), and scopolamine methylbromide (scopolamine MeBr) were obtained from Sigma (St. Louis, MO, USA); tropicamide (1%) was obtained from Bausch & Lomb Pharmaceutical (Tampa, FL, USA). *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 (Rockville, MD, USA). Scintiverse BD was obtained from Fisher Scientific (Pittsburgh, PA, USA). Animal studies were conducted in accordance with the provisions of "Guide for the Care and Use of Laboratory Animals" adopted by the National Institute of Health. Institutional Animal Care and Use Committee (IACUC) approval was obtained prior to the initiation of this research and during its execution.

Preparation of SGa

Both the racemic and isomeric acids SGa were prepared by hydrolysis from the corresponding methyl esters (+)2*R*-SGM and (±)SGM, cyclopentylphenylhydroxyacetoxy-1-methyl-1-methoxycarbonylpyrrolidinium bromide, that have been synthesized and characterized (elemental analysis, nuclear magnetic resonance) in our laboratories as previously reported (30). To 2*R*-SGM and SGM in aqueous solutions, equimolar ratios of 0.1 N NaOH were added. The mixture was stirred at room temperature for 3 h, and completion of reaction was verified by HPLC. After volume adjustment by water, a 1% solution of SGa, or (±)SGa, pH about 6.5, was obtained. The resulting solution was used as is or diluted with normal saline for the experiments.

Analytical Methods

A high-performance liquid chromatographic (HPLC) method was developed for the quantitative analysis of SGa. The system consisted of a Spectra Physics (San Jose, CA, USA) SP 8810 isocratic pump, a SP 8450 UV/Vis detector (wavelength set at 230 nm), an SP 4290 integrator, and a Supelco Discovery C16 column. The mobile phase consisted of acetonitrile, water, and acetic acid at a ratio of 30:70:0.1. At a flow rate of 1 mL min^{-1} and an injection volume of 10 μL, the retention time was 7.10 min, and the detection limit was 1 μg mL^{-1}.

Stability Studies

Stability in Aqueous Solutions

A 0.1% water solution of SGa (pH 6.5) was kept at room temperature or 37°C. At various time points, samples were withdrawn and analyzed by HPLC.

Stability in Biological Media

Freshly collected rat blood, plasma, and 30% liver and lung homogenates were used. Aliquots of 1% of SGa in water solution were added to the biological media at 37°C to yield final concentrations of 0.1%. At appropriate time intervals, samples (0.1 mL) were withdrawn and mixed with 0.2 mL of 5% dimethylsulfoxide in acetonitrile solution. The mixtures were centrifuged, and the supernatants were further diluted two times by water and analyzed by HPLC. The extraction rate was compared to a calibration standard and determined to be 100 ± 3% ($n = 4$).

In Vitro Pharmacodynamic Evaluations

Receptor Binding Affinity

Receptor binding studies on SGa, (±)SGa, glycopyrrolate, and *N*-methylscopolamine were performed with *N*-[^{3}H]methyl-scopolamine (NMS) in assay buffer [phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+}, pH 7.4), following the protocol from Applied Cell Science. A 10-mM NaF solution was added to the buffer as an esterase inhibitor. The assay mixture (0.2 mL) contained 20 μL diluted receptor membranes (receptor proteins: 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 the 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 overnight 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. Detection was performed on a Packard 31800 liquid scintillation analyzer (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 the IC_{50} values (x being the concentration of the tested compound). Based on the method of Cheng and Prusoff (46), 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. Data were analyzed via a nonlinear least-square curve-fitting procedure using Scientist software (MicroMath Inc., Salt Lake City, UT, USA).

Determination of pA_2 Values

Male guinea pigs obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA) and weighing about 400 g were used after overnight fasting. Animals were sacrificed by decapitation, and the ileum (the region of 5 cm upward of the cecum) was isolated and removed. The ileum was cut into 2.5-cm pieces and suspended in an organ bath containing 30 mL of 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., Litchfield, 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 2×10^{-4}–2×10^{-3} M in water solution). Contractions were recorded on a physiograph (Kipp & Zonen Flatbed Recorder, Holland). After maximum response was achieved, the ileum was washed three times, and a fresh Tyrode's solution containing appropriate concentration of the antagonist [SGa, (±)SGa, SGM, glycopyrrolate, or scopolamine] was replaced. An equilibration time of 10 min was allowed for the antagonists before the addition of carbachol. In each experiment, five to six different concentrations were used, and a Schild plot was used to obtain the pA_2 values. Four to six trials were performed for each antagonist.

***In Vivo* Pharmacodynamic Evaluations**

Mydriatic Studies

Topical Administration. The mydriatic effects of SGa were compared to those of glycopyrrolate, tropicamide, and its parent soft drugs, SGM and SGE, in rabbit eyes. Four healthy, male New Zealand white rabbits weighting about 3.5 kg were used. To investigate the dose–mydriatic response relationships, 100 μL of various concentrations of the compounds (0–1%) were administered in their eyes to determine the pharmacodynamically equivalent doses, the lowest doses that induce the maximum pupil dilations. Drug solutions were applied to one eye; only water was applied to the other eye that served as control. Experiments were carried out in a light- and temperature-controlled room. At appropriate time intervals, the pupil diameters of both eyes were recorded. Difference in pupil diameters between each time-point and 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 systemic absorption had occurred or not. The area under the mydriatic response–time curve (AUC^{eff}) was calculated by the trapezoidal rule, and it was used to compare the activity and duration of action of the tested compounds.

Intravenous Administration. New Zealand white rabbits (4 kg) were injected i.v. with SGa or glycopyrrolate at a dose of 2.5 μmol kg^{-1} (about 1 mg kg^{-1}), and the mydriatic response was recorded for both eyes at various time points.

Cardiac Studies

Effect on Resting Heart Rate. Male Sprague–Dawley rats, weighing 300 ± 30 g, were anesthetized with 50 mg kg^{-1} (i.p.) of sodium pentobarbital. Needle electrodes were inserted s.c. into the limbs of the anesthetized rats and were joined to a GOULD 2000 recorder (Gould Inc., Cleveland, OH, USA). Standard leads I, II, and III were recorded at a paper speed of 25 mm s^{-1}. After a 15-min period of stabilization, baseline electrocardiography (ECG) was measured, and drug was administered. Compound SGa in normal saline (5 μmol kg^{-1}, about 2 mg kg^{-1}) or vehicle only was administered in the jugular vein (1 mL kg^{-1}). Heart rate was recorded at designated time-points for 2.5 h.

Effect on Carbachol-Induced Bradycardia. Rats were prepared as previously described. Recording was taken before, during, and after the administration of any of the compounds, until all basic ECG parameters returned to the baseline. ECG recordings were evaluated for the following parameters: PP cycle length (ms) as an indicator of the atrial rate, RR cycle length (ms) as an indicator of the ventricular rate, heart rate (1/min) by the equation of 60,000/RR cycle length, and presence of Mobitz II type atrio–ventricular (A–V) block (2:1, 3:1, etc.). To evaluate the effects of carbachol, the negative chronotropic and dromotropic effects were analyzed. These effects of carbachol were manifested on the surface ECG as sinus bradycardia (lengthening of the PP cycle) and as a development of Mobitz II type A–V block. After ECG recordings were analyzed, the percent changes of heart rate, as compared to that of the baseline, were plotted against time, and the effects of drugs on the percent changes of the heart rate were characterized. Compound SGa (0–5 μmol kg^{-1}) and glycopyrrolate (0.5 μmol kg^{-1}) were dissolved in 0.9% NaCl and injected into the jugular veins (1 mL kg^{-1}) at time 0, while carbachol (80 μg mL^{-1}, 0.06–0.1 mL volume according to the initial individual ECG response of each rat) was injected at various time-points after drug administration. Student's t test was used for statistical evaluations.

Pharmacokinetic Studies

Pharmacokinetics After Intravenous Administration

Male Sprague–Dawley rats (body weight about 400 g) were anesthetized with 30 mg kg^{-1} of sodium pentobarbital

(i.p.). A 1% solution of SGa was injected in the jugular vein. Due to the low sensitivity of HPLC detection (1 μg mL^{-1}), a dose of 30 mg kg^{-1} was used. Blood samples (0.12 mL) were collected through heparinized syringe from the contralateral jugular vein at appropriate time intervals, and plasma (0.05 mL) was separated. The plasma samples were mixed with 0.1 mL of acetonitrile containing 5% dimethyl sulfoxide and centrifuged. The supernatants were further mixed with 1 volume of water, centrifuged, and analyzed by HPLC. Concentrations of SGa were determined via a calibration curve obtained by addition of known amounts of the compound to plasma and prepared following the same methodology for HPLC analysis ($r = 0.995$). Noncompartmental and compartmental pharmacokinetic analysis was performed using WinNonlin (Pharsight Corp., Mountain View, CA USA). In noncompartmental analysis, the area under the curve (AUC) of the plasma concentration *vs.* time was calculated using the trapezoidal rule. The area from the last concentration measured (C_t) to infinity was calculated as C_t / β, where β is the terminal disposition rate constant. Total body clearance (CL_{tot}) was calculated as Dose / AUC. Mean residence time (MRT) was calculated as AUMC / AUC, where AUMC (area under the first moment curve) was calculated using the trapezoidal rule from the graph of plasma concentration × time *vs.* time. Extrapolation of AUMC from the last time point t to infinity was calculated as $C_t / \beta + C_t / \beta^2$. The volume of distribution at the steady state (Vd_{ss}) was determined as the product of CL_{tot} and MRT. For compartmental analysis, data were fitted with a two-compartment model, $C = Ae^{-\alpha t} + Be^{-\beta t}$, where C is the drug concentration in plasma, A and B are the exponential multipliers, and α and β are the hybrid rate constants for the distribution and elimination phase, respectively. AUC was calculated as $(A / \alpha) + (B / \beta)$, and the half-life of the terminal phase ($t_{1/2}$) was obtained as ln 2 / β. The volume of distribution of the central compartment, Vd_c, was calculated as Dose / $A + B$. The elimination rate constant, K_{el}, was calculated as CL_{tot} / Vd_c. Unweighted data were used in all analyses.

Excretion After Intravenous Administration

Male Sprague–Dawley rats (350 ± 10 g) were anesthetized by injection of sodium pentobarbital (30 mg kg^{-1}, i.p.). The urinary tract was closed to prevent urination, and urine samples were collected directly from the urinary bladder through a 26-gauge needle. The peritoneal cavity was exposed, and the bile duct was cannulated using a polyethylene tube (PE 10). SGa (10 mg as 1 mL 1% solution) was administered intravenously (jugular vein). At various time intervals after administration, total bile juice and urine were collected and weighed in centrifuge tubes. Samples were taken at 0 min (control) and then every 15 min until 2 h after i.v. administration. A 5% mannitol in normal saline solution was injected (0.5 mL) in the jugular vein every 30 min to increase the volume of the urine for sample collections (about 0.2 mL per 15 min). The collected bile and urine samples were prepared, diluted properly, and analyzed by HPLC as described in "Pharmacokinetics After Intravenous Administration." Noncompartmental analysis for the urine data after bolus i.v. was performed using WinNonlin. Maximum observed excretion rate (C_{max}) was recorded, and the first-order elimination rate constant (k) was estimated via linear regression using the logarithmic plot. The half-life ($t_{1/2}$) was calculated as ln 2 / k, and the amount of cumulative elimination, A_e, was calculated by summation of the concentration–volume products of each sample. Using the remaining amounts *vs.* time, a sigma (–) plot was also developed, and k and $t_{1/2}$ were calculated subsequently for comparison.

RESULTS AND DISCUSSION

Preparation of SGa

Both SGa and its racemic equivalent (±)SGa are hydrolytic products of previously reported soft anticholinergic compounds, cyclopentylphenylhydroxyacetoxy-1-methyl-1-methoxycarbonylpyrrolidinium bromide, (+)2*R*-SGM or (±)SGM. They were prepared by simple hydrolysis in basic aqueous solution corresponding to the hydrolytic step shown in Fig. 1.

Solubility and Stability

Both zwitterionic SGa and (±)SGa are very soluble in aqueous solutions (pH 6.5) and biological media, and they are also very stable. A 0.1% water solution kept at room temperature showed no decomposition after 1 year. In freshly collected rat blood, plasma, and 30% liver and lung homogenates, there was no change in the HPLC peak area after 2 h at 37°C. Thus, SGa (0.1 %) is also very stable toward metabolic transformations.

Pharmacodynamic Evaluations

Receptor Binding Affinity

The receptor binding affinities, pK_i, of SGa and (±)SGa are presented in Table I together with that of the parent soft drugs, the methyl ester 2*R*-SGM and the ethyl ester 2*R*-SGE, and those of glycopyrrolate and *N*-methylscopolamine for comparison. The receptor binding affinity of the zwitterionic metabolite is considerably less than that of glycopyrrolate or *N*-methylscopolamine, and is about 1 order of magnitude less than that of their parent methyl ester soft drugs—i.e., SGa *vs.* 2*R*-SGM (all differences statistically significant at the $p < 0.05$ level using either t tests or nonparametric Mann–Whitney U tests). This is in good agreement with the hypothesis that the presence of acidic moiety formed by hydrolysis of the parent soft drug ester inactivates the drug, but because in these zwitterionic structures, the electronic distribution somewhat resembles those of the neutral (and active) anticholinergics (Fig. 2), some activity is still retained. Contrary to its parents 2*R*-SGM or 2*R*-SGE that show no M_3/M_2 subtype selectivity, SGa shows a significantly better ($p < 0.01$, t test assuming equal variances) (almost fivefold) subtype selectivity (Table I), further increasing its safety profile. Furthermore, even on these structures, the 2*R* isomer show increased affinity, confirming the stereospecificity of muscarinic receptors. Hill coefficients (n) were not very different from unity, indicating that generally drug–receptor interactions obeyed the law of action and binding took

Table I. Receptor Binding Affinities, M_3/M_2 Selectivities, and pA_2 Values

Compound	Subtypes of Cloned Muscarinic Receptors[a]				Selectivity	
	M_1	M_2	M_3	M_4	M_3/M_2	pA_2^b
SGa (2*R*-SGa)	8.11 ± 0.16 (0.83 ± 0.11)	7.48 ± 0.12 (1.10 ± 0.11)	8.12 ± 0.10 (0.83 ± 0.01)	8.23 ± 0.12 (0.83 ± 0.01)	4.4 ± 0.3	7.20 ± 0.19
(±)SGa	6.19 ± 0.06 (1.11 ± 0.06)	5.48 ± 0.13 (1.02 ± 0.20)	5.84 ± 0.07 (1.01 ± 0.07)	6.44 ± 0.06 (0.84 ± 0.06)	2.3 ± 0.7	6.42 ± 0.30
2*R*-SGM	8.89 ± 0.04 (0.83 ± 0.11)	8.87 ± 0.05 (1.10 ± 0.11)	9.00 ± 0.06 (0.83 ± 0.01)	9.52 ± 0.01 (0.83 ± 0.01)	1.4 ± 0.1	8.31 ± 0.05
2*R*-SGE	8.67 ± 0.16 (0.86 ± 0.08)	8.84 ± 0.34 (0.92 ± 0.01)	8.74 ± 0.02 (1.09 ± 0.15)	8.85 ± 0.13 (0.89 ± 0.02)	0.9 ± 0.6	8.55 ± 0.16
Glycopyrrolate	9.76 ± 0.05 (1.37 ± 0.20)	9.19 ± 0.18 (0.99 ± 0.11)	8.73 ± 0.05 (1.14 ± 0.25)	9.90 ± 0.08 (1.02 ± 0.01)	0.4 ± 0.1	8.57 ± 0.12
Scopolamine methyl bromide	9.69 ± 0.01 (0.92 ± 0.10)	9.18 ± 0.21 (1.02 ± 0.02)	9.29 ± 0.12 (1.07 ± 0.01)	9.92 ± 0.21 (0.90 ± 0.04)	1.3 ± 0.4	9.16 ± 0.19

Data represent mean ± SD.
[a] Receptor binding pK_i data represent mean ± SD of three experiments. Numbers in parentheses denote Hill slopes.
[b] pA_2 values were determined on four to six ileum strips obtained from different animals.

place at only one site (47). The receptor binding affinity of *N*-methylscopolamine determined here was in good agreement with previously published results (29,48,49).

Guinea Pig Ileum Assay, pA_2 Value

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 twofold shift to the right in an agonist's concentration–response curve, are a classical functional study of anticholinergic affinity (at M_3 muscarinic receptors). Values obtained for the present compounds from ileum longitudinal contractions by using carbachol as agonists with the method of van Rossum (50) are presented in Table I. Compared to 2*R*-SGM, 2*R*-SGE, glycopyrrolate, or *N*-methylscopolamine, pA_2 values indicate even somewhat less activity for the zwitterionic SGa than the receptor binding assays, and this assay also confirmed the higher activity of the 2*R* isomers. The pA_2 values of the SGa and (±)SGa metabolites are 1.1 ± 0.3 and 1.3 ± 0.3 less than those of the corresponding ethyl and methyl parent ester soft drugs, respectively, indicating again that they are a good order of magnitude less active (30). Comparison of this to the average of close to 2 orders of magnitude decrease in activity seen previously in the same pA_2 assay for three other acidic metabolites *vs.* their corresponding ethyl ester parents, 1.8 ± 0.5 (31–33), confirms the hypothesis that these spatially close zwitterions are likely to retain more activity than the previous metabolite structures, but are still inactivated to a good extent.

Mydriatic studies

These studies were performed to evaluate the *in vivo* potency of these metabolites following local or systemic administration.

Topical Administration. The potency and duration of action of SGa was compared to those of its parent ester soft drugs 2*R*-SGM and 2*R*-SGE, glycopyrrolate, and tropicamide (the most frequently used short-acting mydriatic agent). Following the topical administration of a 100-μL drug solution to one eye in rabbits, the pupil size was measured, and the maximum mydriatic effect (% change in pupil size) and area under the mydriatic response–time curves ($AUC^{eff}_{0–168\,h}$) were determined (Table II). Accordingly, SGa produces local mydriatic activity (R_{max}), but only with short duration of action ($AUC^{eff}_{0–168\,h}$) (Fig. 3). Recovery times, length of time needed for the size of pupil in the treated eye to recover within less than 1 mm of the control, were approximately 102, 24, and 6 h after administration of 0.2% of glycopyrrolate, 1.0% 2*R*-SGE, and SGa, respectively. SGa was less potent and shorter acting than its parent esters, 2*R*-SGM and 2*R*-SGE. In agreement with previous results, its racemic form (±)SGa showed even lower potency. Furthermore, SGa did not cause any observable irritation reactions, such as eye closing, lacrimation, or mucous discharge; and unlike conventional anticholinergics (21,22), it did not cause pupil dilation in the contralateral, untreated eye as an indication of not only low topical and systemic side effects, but also of rapid elimination from systemic circulation.

Table II. Maximum Response (R_{max}, Maximum % Change in Pupil Size) and Area Under the Response–Time Curve (AUC^{eff}) After Topical Administration (0.1 mL)

Compound	Concentration (%)	R_{max} (%)	$AUC^{eff}_{0–168\,h}$
SGa (2*R*-SGa)	0	0.00 ± 0.00	0 ± 0
	0.01	31.00 ± 7.14	73 ± 24
	0.02	38.79 ± 7.45	103 ± 22
	0.05	51.38 ± 8.81	175 ± 46
	0.1	50.34 ± 7.92	182 ± 40
	0.2	55.65 ± 9.24	240 ± 38
	0.5	56.79 ± 10.71	590 ± 205
	1	53.65 ± 10.84	612 ± 115
(±)SGa	0.01	1.85 ± 2.14	0.7 ± 0.9
	1	45.37 ± 8.19	119 ± 34
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	0.02	35.97 ± 9.84	1879 ± 664
	0.05	48.73 ± 12.66	2476 ± 847
	0.1	52.95 ± 10.93	3732 ± 866
	0.2	53.24 ± 14.49	4923 ± 2175
Tropicamide	0.02	30.27 ± 9.74	99 ± 40
	0.05	35.00 ± 9.18	167 ± 116
	0.2	42.72 ± 9.60	435 ± 150
	0.5	44.64 ± 11.17	622 ± 171

Data represent mean ± SD of four trials.

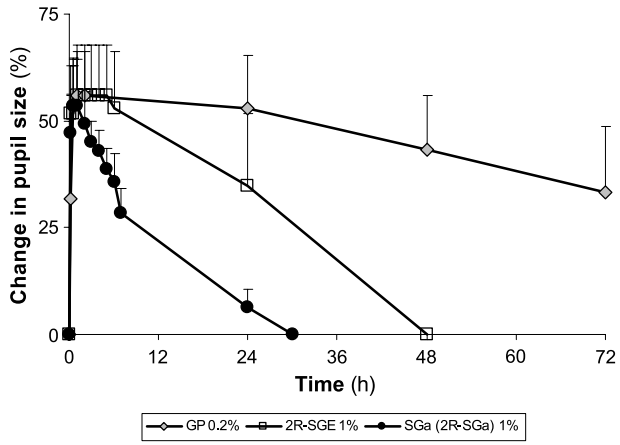

Fig. 3. Mydriatic response after topical administration of SGa, its parent soft drug 2*R*-SGE, and glycopyrrolate in rabbits.

Intravenous Administration. To evaluate the likelihood of causing side effects after systemic administration, the mydriatic response following an intravenous (i.v.) dose of 2.5 μmol kg^{-1} (about 1 mg kg^{-1} through ear vein) was also investigated in rabbits. As shown in Fig. 4, SGa produced some mydriasis after i.v. administration, but its magnitude and duration of action were considerably less than those produced by glycopyrrolate. A related issue raised by the reviewers that should also be discussed here is that of the possibility of side effects caused not only by the acid part (i.e., SGa), but also by the alcohol part formed during metabolism of the parent soft drugs—i.e., methanol for SGM, ethanol for SGE, and so on. The methyl ester, even if not intended for development as the parent soft drug here, is of utmost concern, as the methanol formed during its hydrolysis is the alcohol most likely to cause eye-related side effects. However, the alcohol formed represents only a very small fraction of the administered dose; for example, it represents only about 7% of the administered parent SGM dose (about 0.07 mg kg^{-1} with the 1 mg kg^{-1} dose used here if 100% of the parent is hydrolyzed). Such alcohol concentrations of <0.1 ppm are well below those formed from dietary exposure as proved in the case of aspartame, a readily hydrolyzed dipeptide methyl ester sweetener, where even long-term administration of very high doses (e.g., 75 mg kg^{-1}) caused no persistent changes in methanol blood levels and no observable symptoms (51,52). Various methyl ester drugs (many of them soft drugs) are also on the market and are among the safest drugs used, e.g., esmolol, methylphenidate, or remifentanil. For methanol, the NOAEL (no observable adverse effects level, rat) for chronic oral exposure is 500 mg kg^{-1} day^{-1} (53); hence, the dose levels used here are around 1/10,000th of this NOAEL, and the situation is better in humans, where oral TD_{Lo} values are in the 3500 mg kg^{-1} range (54) and the expected therapeutic doses are smaller than those used in these animal studies. The situation is not significantly altered even following topical administration because, despite its apparent easy accessibility, the eye is in fact well protected by the tear flow, the small volume of the conjuctival sac, and by the permeability barrier imposed by the cornea. Therefore, in most cases, no more than 2% of the medication introduced to the eye is actually absorbed (55,56), the rest being washed away and absorbed through the nasolacrimal duct and the mucosal membranes of the nasal, oropharyngeal, and gastrointestinal tract and acting as a systemically administered dose.

Cardiac Studies

Effect on Resting Heart Rate. After i.v. administration of normal saline (vehicle control) or SGa in pentobarbital-anesthetized rats, the heart rate was recorded every 10 min up to 2.5 h. Results indicate that at a dose of 5 μmol kg^{-1} (about 2 mg kg^{-1}), SGa did not affect the resting heart rate in any of the four animals (Fig. 5), further confirming its slight subtype selectivity.

Effect on Carbachol-Induced Bradycardia. The magnitude of cardiac effects of SGa and glycopyrrolate were assessed by measuring the extent and duration of their bradycardia-protecting activities. Intravenous injection of carbachol at a dose of 16–26 μg kg^{-1} to rats produces a temporary sinus bradycardia and Mobitz II A–V block in a safe and reproducible manner. This can be fully prevented by prior administration of an anticholinergic agent, and the effects of various anticholinergics differ greatly in their extent and duration of action. In this study, various doses (0.25–5 μmol

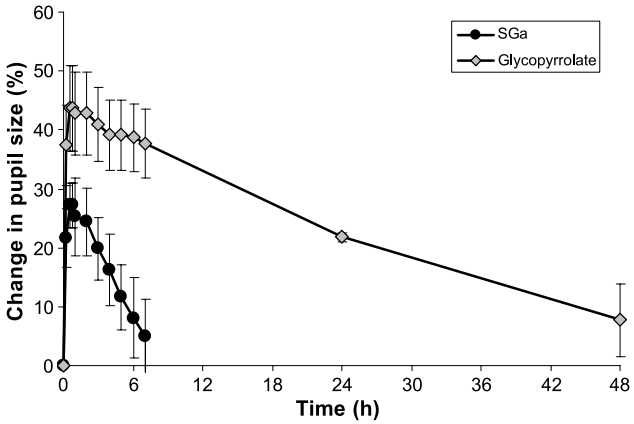

Fig. 4. Mydriatic response after intravenous administration of 2.5 μmol kg^{-1} of SGa or glycopyrrolate in rabbits (n = 4).

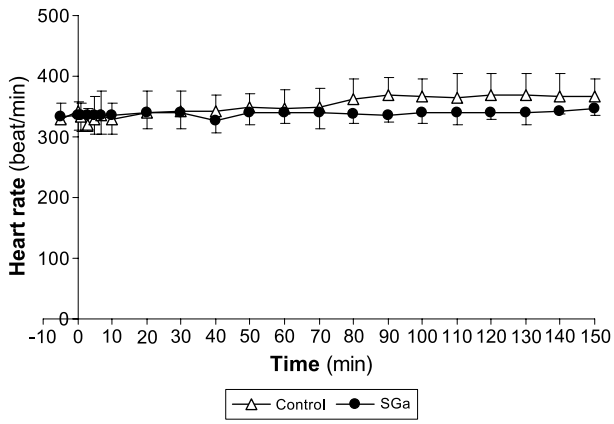

Fig. 5. Effect of SGa (5 μmol kg^{-1}) on the resting heart rate in anesthetized rats (n = 4).

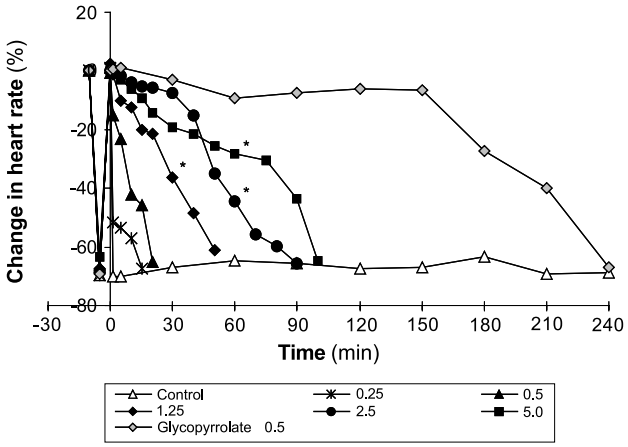

Fig. 6. Protecting effect of SGa (0.25–5 μmol kg^{-1}) and glycopyrrolate (0.5 μmol kg^{-1}) on carbachol induced bradycardia ($n = 4$). *$p < 0.005$ compared to glycopyrrolate.

kg^{-1}) of SGa were investigated and compared to glycopyrrolate (0.5 μmol kg^{-1}). As shown in Fig. 6, carbachol injection (e.g., at −5 min) induced a temporary Mobitz II A–V block with more than 60% inhibition of the normal heart rate (control). After i.v. injection at 0 min of SGa or glycopyrrolate at various doses, carbachol at the same dose induced various degrees of inhibition. The zwitterionic SGa showed bradycardia-protecting activity immediately after i.v. administration, but this diminished rapidly. At a dose of 0.5 μmol kg^{-1}, two out of three rats showed full prevention of the carbachol-induced bradycardia at 1 min, and at a dose of 1.25 μmol kg^{-1}, all three rats showed full prevention. These protective effects disappeared completely in less than 30 and 60 min, respectively. Even at higher doses, such as 2.5 and 5 μmol kg^{-1}, the effect of SGa disappeared in less than 100 min, indicating that fast elimination from the systemic circulation rapidly reduces the potential to induce heart-related side effects. For comparison, glycopyrrolate showed full protection for more than 2.5 h and partial effect for another 1.5 h even at a 10 times smaller dose (0.5 μmol kg^{-1}) (Fig. 6).

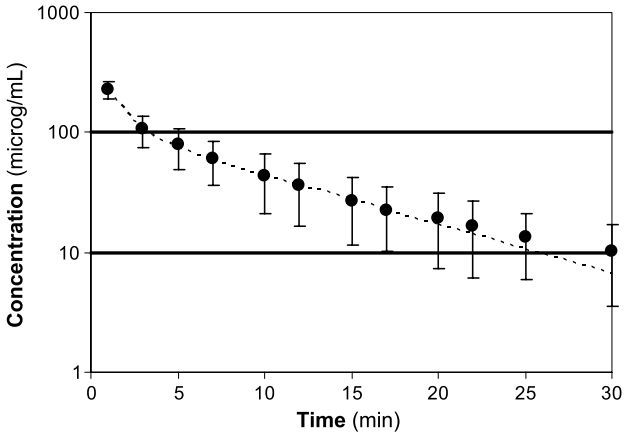

Fig. 7. Mean plasma concentration–time profile after intravenous injection of SGa at a dose of 30 mg kg^{-1} in rats ($n = 4$). The line represents data predicted by the two-compartment model (Table III).

Table III. Pharmacokinetics of SGa After i.v. Bolus Administration in Rats

Parameter	Mean	SD
Dose (mg kg^{-1})	30	
Noncompartmental analysis results		
C_{max} (μg mL^{-1})	330.74	31.96
k_e (min^{-1})	0.066	0.01
$t_{1/2}$ (min)	10.7	1.40
AUC_∞ (μg min mL^{-1})	1676	637
CL_{tot} (mL min^{-1} kg^{-1})	19.59	6.05
$AUMC_\infty$ (μg min^2 mL^{-1})	18,281	10,850
MRT_∞ (min)	10.33	2.12
Vd_{ss} (mL kg^{-1})	192.99	30.94
Compartmental analysis results		
A (μg mL^{-1})	292.46	25.37
B (μg mL^{-1})	111.41	39.71
α (min^{-1})	0.851	0.145
β (min^{-1})	0.094	0.012
$t_{1/2\alpha}$ (min)	0.832	0.14
$t_{1/2\beta}$ (min)	7.44	0.96
Vd_c (mL kg^{-1})	75.20	9.86
Vd_{ss} (mL kg^{-1})	167.91	22.76
r	0.9994	0.0005

MRT: mean residence time, AUC: area under the curve, AUMC: area under the first moment curve, CL_{tot}: total body clearance, Vd_{ss}: volume of distribution at the steady state, Vd_c: volume of distribution of the central compartment.

Pharmacokinetic Studies

Pharmacokinetics After Intravenous Administration

An *in vivo* pharmacokinetic evaluation of SGa was performed in rats. After a single i.v. bolus injection, plasma concentrations at predetermined time points were quantified by HPLC. The observed plasma concentration time profile of SGa (Fig. 7) could be best described by a two-compartment body model corresponding to a biexponential equation, $C = Ae^{-\alpha t} + Be^{-\beta t}$. Pharmacokinetic parameters obtained from

Table IV. Excretion of SGa in Urine After i.v. Bolus Administration in Rats

Parameter	Mean	SD
Dose (mg)	10	
Log rate plot		
C_{max} (mg mL^{-1})	0.193	0.046
A_e (cumm. excr.) (mg)	5.08	0.46
k_e (min^{-1})	0.050	0.0062
$t_{1/2}$ (min)	14.02	1.73
AUC_{0-last} (mg mL^{-1} min)	4.36	0.34
AUC_∞ (mg mL^{-1} min)	4.46	0.31
r	0.974	0.021
Sigma minus plot		
k_e (min^{-1})	0.0539	0.0089
$t_{1/2}$ (min)	13.13	2.22
r	0.995	0.003

noncompartmental (NCA) and compartmental analyses are shown in Table III. The elimination half-life $t_{1/2}$ was about 10.7 min with a volume of distribution Vd_{ss} of 193 mL kg^{-1} and a total body clearance CL_{tot} of about 20 mL min^{-1} kg^{-1} (NCA). For compartmental analysis, the correlation coefficient, r, was >0.999 in all individuals. Parameters were in general agreement with the NCA results. These clearly demonstrate that even a high dose (30 mg kg^{-1}) of SGa is rapidly cleared and well tolerated in animals, an important requirement for soft drug metabolites.

Excretion After Intravenous Administration

Total bile juice and urine were collected every 15 min for up to 2 h and analyzed by HPLC after i.v. administration of 10 mg SGa (1 mL of 1% solution). No detectable levels of SGa were excreted in the bile, but relatively large amounts of SGa were excreted in the urine. Table IV presents the results of noncompartmental analysis of the urine data (log rate plot and sigma minus plot). At 1 h after injection, the cumulative excretion amount was about 50% of the administered dose. The first-order elimination rate constants k_e were estimated, and the elimination half-lives $t_{1/2}$ were derived from the slopes of these two plots (14.0 and 13.1 min, respectively). These results, again, indicate a rapid elimination of SGa from the systemic circulation mainly through urinary excretion.

CONCLUSION

The present PK/PD studies demonstrated that the zwitterionic metabolite SGa retains some, but significantly reduced activity of its parent quaternary ammonium ester soft drugs, and is very rapidly eliminated from the systemic circulation mainly through urinary excretion of its unchanged form. Furthermore, as SGa also showed moderate M_3/M_2 muscarinic receptor subtype selectivity, the likelihood of cardiac side effects is further reduced for this metabolite.

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