Design, Pharmacokinetic, and Pharmacodynamic Evaluation of a New Class of Soft Anticholinergics

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Purpose. To design and evaluate a new class of soft anticholinergics with subtype selectivity.

Methods. A new class of soft anticholinergics was designed based on the "inactive metabolite" approach. Four compounds were synthesized. The potency and soft nature of the compounds were evaluated by receptor binding, cardiac, and mydriatic studies. Stability and pharmacokinetic studies were also performed on these newly synthesized soft anticholinergics.

Results. Receptor binding studies of the soft anticholinergics on cloned muscarinic receptors indicated p*K*i values in the range of 7.5 to 8.9. Two compounds, 9a and 13a, of the series showed muscarinic subtype receptor selectivity (M_3/M_2). In mydriatic studies, 13a and 13b showed shorter duration of action in the treated eyes than tropicamide. In the control eyes, significant dilation of pupils was found only in rabbits treated with atropine and tropicamide, indicating that the soft anticholinergics lack systemic effects because of their facile hydrolytic deactivation. Consistent with their soft nature, this new class of soft anticholinergics displayed much shorter cardiovascular effects in the carbachol-induced bradycardia (10 to 15 min) in rats than atropine (> 60 min). Stability and pharmacokinetic studies suggested that the new soft anticholinergics were rapidly eliminated from plasma (systemic circulation) after i.v. administration.

Conclusions. A new class of anticholinergics was designed and synthesized, and the PK/PD evaluation confirmed they were potent "soft" anticholinergics; two of them showed muscarinic receptor subtype selectivity (M_3/M_2).

KEY WORDS: soft drugs; anticholinergic agents; receptor binding; muscarinic receptor subtypes; metabolism.

INTRODUCTION

Anticholinergics, such as atropine and scopolamine, have been used clinically for the treatment of a variety of disorders (1), predominantly to inhibit the parasympathetic nervous system. Anticholinergics find use in the management of peptic ulcer, as mydriatic/cycloplegic agents, as adjuncts to anesthetic medications, and as antiperspirants. However, the usefulness of anticholinergics is limited by their unwanted toxic effects. For instance, the use of anticholinergics as premedication in anesthetic practice often results in cardiac side effects (2–5); even topical application of anticholinergics can lead to systemic toxicity because of the absorption and drainage of the anticholinergics into the systemic circulation. For example, at least six deaths have been attributed to the ocular administration of atropine (6). Various psychic disturbances have been reported to the application of atropine (7), scopolamine (8), and cyclopentolate (9) as mydriatic/cycloplegic agents. Severe cardiac dysfunction has been reported with the use of atropine as a perioperative mydriatic/cycoplegic agent (10,11). That application is particularly dangerous to the elderly because older people are more likely to suffer from cardiac diseases (11,12). Anticholinergics have been evaluated for their use as antiperspirants for a long time (13–15); however, because of the risk of systemic toxicity when overdosed, it was suggested that these agents should be used under direct physician supervision and not be available for over-the-counter sale (16).

In order to separate the desired therapeutic effects of anticholinergics from their undesired toxic effects, two major approaches have been used. The first of these is the pharmacokinetic (retrometabolic) approach, which includes the development of "soft" anticholinergics to minimize systemic toxicity of anticholinergics (17–20). Briefly, based on specific design concepts, structural analogues of known anticholinergics are designed to include an easily metabolizable moiety in the structure that assures facile deactivation of the compounds when they reach the circulatory system, after they have achieved their therapeutic role. Soft anticholinergics are primarily designed for topical application. Two distinctly different design approaches have been applied: the soft analogue (20,21) and the inactive metabolite approaches (22–26). And one of these compounds, tematropium methyl sulfate, the methyl sulfate of the ethyl ester of [(±)-(carboxyl)-8-methyl-8-azabicycl (3.2.1)oct-3-yl-benzene acetate], based on the inactive metabolite approach, has successfully reached phase II human trials as a short-acting mydriatic agent.

The second approach is the pharmacodynamic approach: development of muscarinic receptor subtype-selective antagonists to minimize toxicity. In parallel with our efforts directed toward the development of safer anticholinergics by soft drug approaches, considerable research has been directed toward the delineation of muscarinic receptors and receptor subtypes in the past 20 years. This was done in the hope that by understanding the function of muscarinic receptors and receptor subtypes, muscarinic antagonists selectively targeted to particular symptom(s) can be made, and therefore, the therapeutic index can be improved (27). Three mammalian muscarinic receptor subtypes have been located and characterized by biochemical and functional studies. Of the three major subtypes (M_1, M_2, M_3), each one has its differential location (e.g., M_1 in brain and M_2 in heart) and is responsible for a specific action; for example, M_1 relates to central nervous system (CNS) function, M_2 triggers cardiac effects, and M_3 stimulates smooth muscle contraction (27). With advances in molecular pharmacology, five muscarinic receptor subtypes (m_1, m_2, m_3, m_4, and m_5) have been cloned from human tissue (28–30) and have been readily available since the early 1990s. The cloned human muscarinic receptor subtypes (m_1 to m_3) have been shown to correlate very well to the ones derived from mammalian tissues (31,32).

Cloned receptors have been used to determine the potency of novel compounds and to determine the subtype se-

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ABBREVIATIONS: SASS: soft anticholinergics with muscarinic receptor subtype selectivity; [^{3}H]NMS, N-[^{3}H]methylscopolamine.

lectivity of antimuscarinic agents (33–35). With the help of modern receptor pharmacology in the past 15 years, significant progress has been made in the search for muscarinic antagonists with subtype selectivity. Currently, several conventional ("hard") muscarinic receptor subtype-selective agents are in advanced clinical trials (36,37), and a few of them such as pirenzepine and AF-DX 116 have been approved for clinical use (37). In order to take advantage of the latest development in receptor pharmacology, a validated receptor-binding method based on commercially available cloned human receptor subtypes has been specifically developed for the fast screening of soft anticholinergics in our laboratory, and a quantitative structure–activity relationship (QSAR) has been established based on the affinity of the soft anticholinergics toward the muscarinic receptor subtypes (38).

The main objective of the present study is to apply the molecular receptor pharmacology technique to our search for a new class of anticholinergics: soft anticholinergics with muscarinic receptor subtype selectivity (SASS). Because both pharmacokinetic and pharmacodynamic approaches are used in the design of SASS, it is expected that this new class of soft anticholinergics is safer than the ones derived from either approach alone. In addition to being used topically as safer mydriatics, antiperspirants, or antiulcer agents, SASS can be used intravenously as a safer preoperative medication for inhibiting excessive secretion. The M_2-mediated cardiac side effects that prevent the conventional anticholinergics such as atropine and glycopyrrolate from being safely administered to cardiac patients (2,39,40) are expected to be minimal because of the subtype selectivity (M_3/M_2) of SASS. And the soft nature of the compounds will allow us to determine the needed dose during the anesthetic procedure.

Design

N-Alkyl-nortropine esters of 2-phenyl-2-cyclohexenecarboxylic acid (I) (Fig. 1) were originally synthesized as bronchodilator agents, some of which showed high anticholinergic activity (41). Receptor binding and functional studies indicated that one of these compounds, (±)-3{[(2-phenylcyclohexene-1-yl)carbonyl]oxy}-8,8-diethyl-8-azoniabicyclo[3.2.1]octane iodide, is a potent and selective antagonist of the M_3-receptor subtype (42). In order to design the SASS based on this group of agents (I), the inactive metabolite approach (20,43) for the design of soft drugs was adapted. A hypothetical metabolite II (Fig. 1) was chosen as the lead compound for the design of the soft drugs; maintaining the positively charged quaternary nitrogen would minimize the central nervous system (CNS) toxic effects because of lack of penetration of the blood–brain barrier. The soft drugs (III) designed are expected to be rapidly hydrolyzed to II *in vivo* by ubiquitous esterases. Another hypothetical metabolite, IIa, is the positional isomer of II. It is known that the smaller size of the equatorial N-substituent of N-alkyl-nortropine esters of 2-phenyl-2-cyclohexenecarboxylic acids is associated with higher potency (41). Thus, it is expected that the soft drugs based on IIa as the lead will be more potent than those based on II. The hypothetical metabolites are expected to be highly polar and ionized at physiologic pH and thus be subject to facile elimination from the systemic circulation either directly or after conjugation. On the other hand, because strong

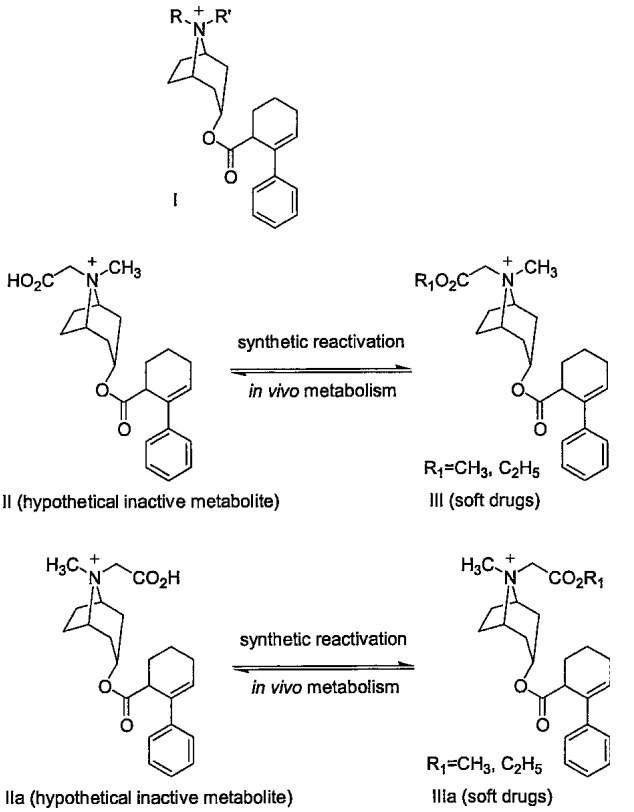

Fig. 1. Design of a new class of soft anticholinergics.

nucleophilic groups are shown to be present at the muscarinic receptor site (44), the zwitterionic metabolites (II or IIa) that result from the hydrolysis of the soft drugs will have an unfavorable interaction with the receptor site; thus, they are expected to be less active than the parent soft drugs (22,23).

MATERIALS AND METHODS

All chemicals used were reagent grade. N-[^{3}H]Methylscopolamine was obtained from Dupont NEN Research (Boston, MA). Atropine was from Sigma Chemicals Co. (St. Louis, MO). Tropicamide was from Schein Pharmaceutical (Florham Park, NJ). All other chemicals were from Aldrich Chemical Company (Milwaukee, WI). Scintiverse BD and other solvents were from Fisher Scientific Co. (Pittsburgh, PA). All melting points were recorded using Fisher-Johns melting point apparatus and were uncorrected. NMR data were recorded with Varian 300 NMR spectrometer and were reported in parts per million (δ) relative to tetramethylsilane. All compounds were dissolved in $CDCl_3$. Elemental analysis was carried out at Atlantic Microlab Inc. (Atlanta, GA). Thin-layer chromatography was carried out using an EM Science DC-Plastic foil plate coated to a thickness of 0.2 mm with silica gel 60 containing fluorescent (254) indicator. Column chromatography was performed with silica gel (70–230) on an appropriate mobile phase. All the animal studies were conducted in accordance with the guidelines set forth in the Declaration of Helsinki and the "Guiding Principles in the Care and Use of Animals" (DHEW Publication, NIH 80-23).

Synthesis

The pathways of synthesis of soft drugs are presented in Fig. 2 and 3. and Syntheses of 1– 4 were performed by fol-

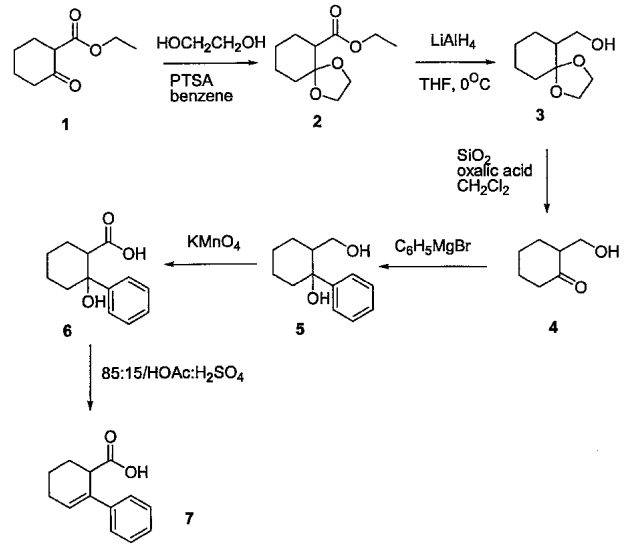

Fig. 2. Synthesis of 2-phenylcyclohex-2-enecarboxylic acid.

lowing the method of Hitchcock *et al.* (45), and the syntheses of 5–6 were accomplished by appropriate modifications of the method of Zimmerman *et al.* (46). The synthesis of compound 7 (2-phenylcyclohex-2-enecarboxylic acid) is done as follows (41): 1 g of 6 (4.5 mmol) was added to a mixture of sulfuric acid (1.5 ml) and acetic acid (8.5 ml) with stirring at room temperature. The mixture was stirred for another 15 min to dissolve all solids and then poured into 100 ml of ice water with stirring. After white crystallized solids appeared, the mixture was continuously stirred for another 30 min. After

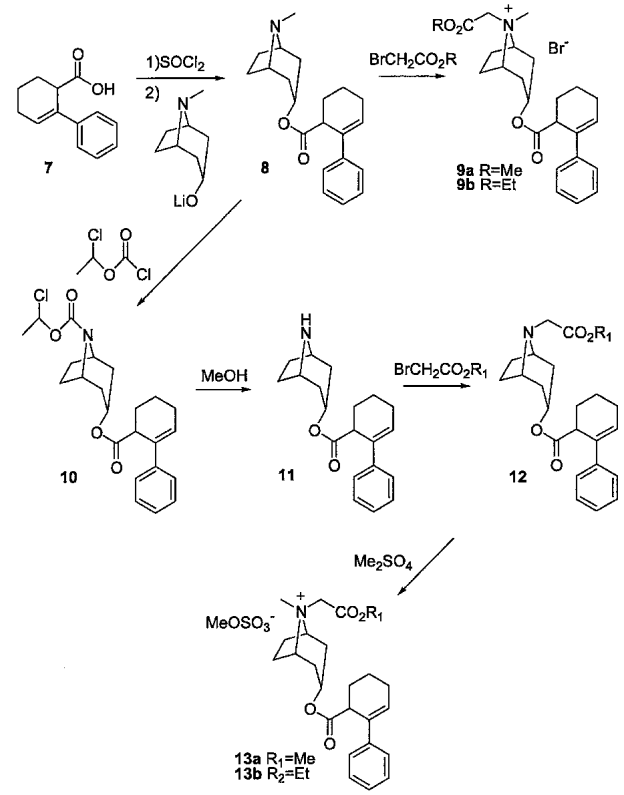

Fig. 3. Synthesis of 9a, 9b, 13a, and 13b.

storage in a –20°C freezer overnight, the above mixture was filtered to give 7 as a white solid (0.75g, 83%).

^{1}H-NMR ($CDCl_3$): 1.71–2.56 (6H, m, $3CH_2$, cyclohexyl), 3.69 (1H, br, $CHCO_2$), 6.22 (1H, t, 3-H), 7.20-7.33 (5H, m, ph) ppm.

Synthesis of 8 (8-aza-8-methylbicyclo [3.2.1]oct-3-yl 2-phenylcyclohex-2-enecarboxylate (a colorless liquid) was achieved through appropriate modifications of the method of Juhasz *et al.* (25).

^{1}H-NMR ($CDCl_3$): 1.20-2.27 (14H, m, $3CH_2$ cyclohexenyl, $4CH_2$, tropyl), 2.26 (3H, s, NCH_3), 2.98-3.10 (2H, brd, tropyl 1, 5-H), 3.70 (1H, s, $CHCO_2$), 4.87 (1H, tropyl 3-H CHO), 6.22 (1H, t, $CH=CC_6H_5$), 7.25–7.34 (5H, m, ph) ppm.

The synthesis of methyl-2-[8-aza-endo-8-methyl-3-(2-phenylcyclohex-2-enylcarbonyloxy)bicyclo[3.2.1]oct-8-yl]acetate, bromide (9a) and ethyl 2-[8-aza-endo-8-methyl-3-(2-phenylcyclohex-2-enylcarbonyloxy)bicyclo[3.2.1]oct-8-yl]acetate, bromide (9b) was done as follows: To 2 g (6.14 mmol) of 8 in 20 ml of anhydrous acetonitrile, 15.36 mmol of methyl bromoacetate or ethyl bromoacetate was added. The above mixture was stirred under argon for 19 h, which evaporated acetonitrile to generate an oily substance, which was further purified by precipitation with CH_2Cl_2/ethyl ether to give pure 9a or 9b as a white solid.

9a: (2.4 g, 81%). M.P. 170-171°C. ^{1}H-NMR ($CDCl_3$): 1.26–2.28 (6H, m, $3CH_2$, cyclohexenyl), 2.63–2.69 (8H, m, $4CH_2$, tropyl), 3.57 (3H, s, NCH_3), 3.78 (4H, s, OCH_3 and 1-H of cyclohexenyl), 4.59–4.69 (2H, br d, tropyl's 1,5-H), 4.84 (2H,s, NCH_2), 5.05 (1H, t, CHO), 6.28 (1H, t, 2-H of cyclohexenyl), 7.23–7.33 (5H, m, Ph) ppm. Elemental analysis ($C_{24}H_{32}O_4NBr$): calculated/found (%); C, 60.25/60.06; H, 6.74/6.81; N, 2.92/2.87.

9b: (2.2 g, 73%). M.P.186–187°C. ^{1}H NMR ($CDCl_3$): 1.26–2.60 (14H, m, $3CH_2$ cyclohexenyl, $4CH_2$ tropyl), 1.31 (3H, t, CH_2CH_3), 3.37 (3H, s, NCH_3), 3.76 (1H, t, 1-H of cyclohexenyl), 4.22 (2H, q, CH_2CH_3), 4.58, 4.67 (2H, br d, tropyl's 1,5-H), 4.82 (2H, s, NCH_2), 4.95 (1H, t, CHO), 6.22 (1H, t, 2-H of cyclohexenyl), 7.20–7.31 (5H, m, Ph) ppm. Elemental analysis ($C_{25}H_{34}O_4NBr$): calculated/found (%); C, 60.97/61.10; H, 6.55/6.96; N, 2.84/2.79.

The synthesis of 11, 8-azabicyclo[3.2.1]oct-3-yl 2-phenylcyclohex-2-enecarboxylate, proceeded as follows. To 1 g (3.07 mmol) of 8 in 10 ml of 1,2-dichloroethane, 1.09 g (7.63 mmol) of 1-choroethyl chloroformate in 5 ml of 1,2-dichoroethane was added dropwise at 0°C. The mixture was then refluxed for 1 h. Residue from evaporation of the solvent of the reaction mixture was then refluxed in methanol for 45 min. Removal of methanol under reduced pressure gave 11 as a solid (0.93g, 97%).

^{1}H NMR ($CDCl_3$): 1.46–2.25(6H, m, $3CH_2$, cyclohexenyl), 2.66–2.90 (8H, m, $4CH_2$, tropyl), 3.71 (1H, s, 1-H of cyclohexenyl), 3.80–3.85 (2H, br d, tropyl's 1,5-H), 4.95 (1H, t, CHO), 6.23 (1H, t, cyclohexenyl's 3-H), 7.23–7.36 (5H, m, Ph) ppm.

The synthesis of methyl-2-[8-aza-3-(2-phenylcyclohex-2-enylcarbonyloxy)bicyclo[3.2.1]oct-8-yl]acetate (12a) and ethyl-2-[8-aza-3-(2-phenylcyclohex-2-enylcarbonyloxy)bicyclo[3.2.1]oct-8-yl]acetate (12b) was done as follows: To well-stirred compound 11 (1 g, 3.21 mmol) in 20 ml of N,N-dimethyl formamide (DMF) with 1 g of K_2CO_3 was added 3.21 mmol of methyl bromoacetate or ethyl bromoac-

etate. The mixture was stirred under argon for 20 h. Then the DMF was removed under reduced pressure. The residue was added with 5 ml of saturated $NaHCO_3$ solution, which was then extracted three times with ethyl ether to give crude 12a or 12b. The above crude products were further purified by flash chromatography on ethyl acetate to give pure 12a or 12b as colorless oil.

12a (0.95g, 75%). ^{1}H NMR ($CDCl_3$): 1.20–1.80 (6H, m, $3CH_2$, cyclohexenyl), 1.81–2.20 (8H, m, $4CH_2$, tropyl), 3.01–3.06 (2H, br d, tropyl's 1,5-H), 3.06 (2H, s, NH_2) 3.63 (4H, s, OCH_3 and 1-H of cyclohexenyl), 4.82 (1H, m, OCH), 7.12–7.28 (5H, m, Ph) ppm.

12b (0.97g, 76%). ^{1}H-NMR ($CDCl_3$): 1.23–1.70 (6H, m, $3CH_2$, cyclohexenyl), 1.71–2.27 (8H, m, $4CH_2$, tropyl), 1.25 (3H, t, CH_3), 3.01-3.15 (2H, br d, tropyl's 1,5-H), 3.72 (1H, t, 1-H of cyclohexenyl), 4.15–4.17 (2H, q, CH_2CH_3), 4.89 (1H, m, OCH), 7.25–7.35 (5H, m, Ph) ppm.

The synthesis of methyl 2-[8-aza-exo-8-methyl-3-(2-phenylcyclohex-2-enylcarbonyloxy)bicyclo[3.2.1]oct-8-yl]acetate, methyl hydroxysulfonate (13a) and ethyl 2-[8-aza-exo-8-methyl-3-(2-phenylcyclohex-2-enylcarbonyloxy) bicyclo[3.2.1]oct-8-yl]acetate, methyl hydroxysulfonate (13b) was carried out as follows: To compound 12 (a or b, 2.50 mmol) in 10 ml of anhydrous acetonitrile was added dimethyl sulfate (0.788 g, 6.25 mmol). The mixture was stirred at room temperature for 15 h, and then acetonitrile was removed under reduced pressure. The residue was purified by precipitation (methylene chloride/ethyl ether) to give 13a or 13b as white solids.

13a (1.15 g, 90%): M.P.150–151°C. ^{1}H NMR ($CDCl_3$): 1.25–2.70 (14H, m, $3CH_2$ cyclohexenyl, $4CH_2$ tropyl), 3.18 (3H, s, NCH_3), 3.66 (3H, s, CH_3SO_4), 3.71(1H, t, 1-H of cyclohexenyl), 3.77 (3H, s, OCH_3), 4.35–4.45 (2H, br d, tropyl's 1,5-H), 4.70 (2H, s, NCH_2), 5.03 (1H, t, OCH), 7.26–7.31 (5H, m, Ph) ppm. Elemental analysis ($C_{25}H_{35}O_8NS$): calculated/found (%); C, 58.92/58.97; H, 6.92/6.86; N, 2.75/2.45.

13b (1.19 g, 91%): M.P.169–170°C. ^{1}H NMR ($CDCl_3$): 1.25–2.60 (14H, m, $3CH_2$ cyclohexenyl, $4CH_2$, tropyl), 3.24(3H, s, NCH_3), 3.72 (4H,s, CH_3SO_4 and 1-H of cyclohexenyl), 4.24 (2H, q, CH_3CH_2O), 4.40–4.54 (2H, br d, tropyl's 1,5-H), 4.60 (2H, s, NCH_2), 5.01 (1H, m, OCH), 6.30 (1H, t, cyclohexenyl 1-H), 7.21–7.40 (5H, m, Ph) ppm. Elemental analysis ($C_{26}H_{37}O_8NS$): calculated/found (%); C, 58.82/58.77; H, 7.02/7.08; N, 2.64/2.60.

Receptor Binding Studies

Binding studies and data analyses were performed by following previously published methods (38).

Mydriatic Studies

Mydriatic studies were conducted by adapting the previously published methods (47).

Cardiac Studies

Studies were performed by appropriate modification of previously published methods (25).

Stability Studies

Analytic Methods

A high-performance liquid chromatographic method was developed to detect the soft drugs. The system consisted of a Spectra Physics (San Jose, CA) SP 8810 isocratic pump, a SP 8450 uv/vis detector with wavelength set to 254 nm, and SP 4290 integrator. A Supelcosil LC ABZ column (Supelco, Bellofonte, PA) was used. The mobile phase consisted of acetonitrile and water (40:60), including 0.1% octanesulfonic acid, 0.2% acetic acid, and 0.1% tetrahydrofuran (THF). At a flow rate of 1.5 ml/min, the retention times of 9a, 9b, 13a, and 13b were 3.63, 5.14, 3.91, and 5.14 min, respectively. With an injection volume of 20 μl, the detection limits of the compounds were 1 μg/ml.

Stability in Biologic Media

Methods were adapted from Huang *et al.* (47).

Pharmacokinetic Studies

Rats (300 to 400 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg). 13a (dissolved in 20% (w/w) of 2-hydroxypropyl-β-cyclodextrin solution) was injected into the jugular vein, over 1 min, at doses of 5, 10, and 15 mg/kg and a dosing volume of 1 ml/kg. For the data treatment (as bolus injection), the midtime of the injection was used as 0 time. Blood samples, 0.1 ml, were collected through the jugular vein at appropriate time intervals for 150 min. The samples were mixed immediately with 0.2 ml of acetonitrile containing 5% dimethyl sulfoxide solution to halt further enzymatic hydrolysis in the blood, and then centrifuged. Subsequently, the samples were injected in the HPLC for the determination the concentrations of soft drugs. The concentration of 13a was determined using a calibration curve developed by adding known amounts of the compound to the blood ($r = 0.995$) and prepared as samples. Noncompartmental and compartmental pharmacokinetic analysis: in noncompartmental analysis, the area under the curve, AUC, of the blood concentration vs. time was calculated using the trapezoidal rule. The area from the last measurement, C_t, to infinity was calculated as C_t/β, where β was the terminal disposition rate constant. The total body clearance, Cl_{tot}, was calculated as dose/AUC. Mean resident time, MRT, was calculated as AUMC/AUC, where AUMC, the area under the first moment curve, was calculated using the trapezoidal rule from the curve of blood concentration × time vs. time, and the area 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 calculated as Cl_{tot} multiplied by MRT. For compartmental analysis, a pharmacokinetic analysis program (PK-Analyst, Micromath, Salt Lake City, UT) was used. The results were best fitted into 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 constant in the central compartment and peripheral compartment, respectively. The AUC was calculated as $A/\alpha + B/\beta$, and the half-life of the terminal phase, $t_{1/2}$, was calculated as $\ln 2/\beta$. The volume of distribution of the central compartment, V_{dc} was calculated as dose/(A + B); the volume of distribution during the elimination phase, V_{darea} was cacu-

lated as CL_{tot}/β, and the elimination rate constant, K_{el}, was calculated as CL_{tot}/V_{dc}.

RESULTS

Synthesis

The synthesis of the 2-phenylcyclohex-2-enecarboxylic acid (**7**) intermediate is shown in Fig. 2; the subsequent syntheses of the soft drugs 9a, 9b, 13a, 13b are presented in Fig. 3. 8-Aza-8-methylbicyclo [3.2.1]oct-3-yl 2-phenylcyclohex-2-enecarboxylate (8) was synthesized based on the methods of Crowther *et al.* (48) and Juhasz *et al.* (25) by reacting 2-phenylcyclohex-2-enecarboxylic chloride with the lithium salt of tropine. The syntheses of methyl-2-[8-aza -8-methyl-3-(2-phenylcyclohex-2-enylcarbonyloxy)bicyclo[3.2.1]oct-8-yl]acetate, bromide (9a) and ethyl 2-[8-aza-endo-8-methyl-3-(2-phenylcyclohex-2-enylcarbonyloxy)bicyclo[3.2.1]oct-8-yl]acetate, bromide (9b) were accomplished by quaternizing 8 with the corresponding bromoacetyl ester. It is well established that the configuration at the ring nitrogen atom in the quaternary tropane derivatives depends on the sequence in which the individual alkyl groups are introduced (49,50). The alkyl group that entered last has been shown to enter the equatorial position. The syntheses of the isomeric methyl 2-[8-aza-3-(2-phenylcyclohex-2-enylcarbonyloxy)bicyclo[3.2.1]oct-8-yl]acetate (12a) and ethyl 2-[8-aza-3-(2-phenylcyclohex-2-enylcarbonyloxy)bicyclo[3.2.1]oct-8-yl]acetate (12b) were less straightforward. First, we attempted to react 2-phenylcyclohex-2-enecarboxylic chloride with the lithium salt of alkoxyl 2-[8-aza-3-bicyclo[3.2.1]oct-8-yl]acetate. However, because of the lability of the alkoxyl 2-[8-aza-3-bicyclo[3.2.1]oct-8-yl]acetate group, the lithium salt of alkoxyl 2-[8-aza-3-bicyclo[3.2.1]oct-8-yl]acetate could not be made. The syntheses of 12a and 12b were eventually accomplished by adapting the method of Koreeda and Lueng (51) through the initial demethylation of 8 followed by the introduction of the alkyoxycarbonylmethyl group. Quaternization of 12a and 12b with dimethyl sulfate produced the soft drugs methyl 2-[8-aza-exo-8-methyl-3-(2-phenylcyclohex-2-enylcarbonyloxy)bicyclo[3.2.1]oct-8-yl]acetate, methyl hydroxysulfonate (13a) and ethyl 2-[8-aza-exo-8-methyl-3-(2-phenylcyclohex-2-enylcarbonyloxy)bicyclo[3.2.1]oct-8-yl]acetate, methyl hydroxysulfonate (13b).

In Vitro Pharmacodynamic Evaluation: Receptor Binding Studies

Receptor binding studies using m_1, m_2, m_3, and m_4 subtypes were performed on the soft anticholinergics 9(a, b) and 13(a, b). The results are summarized in Table I. It is shown that the p*K*i values ranged from 7.5 to 8.9, indicating comparable potency to the lead compound. The results demonstrated that compounds 9a and 13a displayed muscarinic subtype selectivity (M_3/M_2). In addition, compound 9a also showed M_3/M_1 selectivity. Based on QSAR studies, Turbanti *et al.* (41) established that smaller size of the equatorial N-substituent of an N-alkyl-nortropine esters of 2-phenyl-2- cyclohexenecarboxylic acids, was favored for higher potency. By their arguments, 13a of our series should be more potent than the other soft anticholinergics. However, this was not the case. There was no significant difference in the potency between the methyl- or ethylester soft anticholinergics. Also, there was no significant difference between the potency of the endo and exo isomers.

Table I. Receptor Binding Values* for 9(a,b) and 13(a,b)

Compound	Receptor subtype			
	m_1	m_2	m_3	m_4
Atropine	9.08 ± 0.12	9.04 ± 0.20	9.28 ± 0.07	9.50 ± 0.04
	(0.98 ± 0.03)	(1.01 ± 0.02)	(0.96 ± 0.02)	0.101 ± 0.04)
9a	7.86 ± 0.03	7.73 ± 0.10	8.99 ± 0.01	8.43 ± 0.07
	(0.78 ± 0.05)	(0.91 ± 0.10)	(0.81 ± 0.02)	(0.90 ± 0.02)
9b	7.93 ± 0.04	7.97 ± 0.03	8.64 ± 0.05	8.20 ± 0.06
	(0.86 ± 0.01)	(0.88 ± 0.03)	(0.87 ± 0.06)	(0.91 ± 0.07)
13a	7.89 ± 0.07	7.38 ± 0.07	8.49 ± 0.02	8.11 ± 0.06
	(0.83 ± 0.05)	(1.07 ± 0.07)	(0.81 ± 0.04)	(1.07 ± 0.02)
13b	7.98 ± 0.04	7.70 ± 0.06	8.62 ± 0.05	8.17 ± 0.03
	(0.91 ± 0.05)	(0.80 ± 0.06)	(0.87 ± 0.05)	(0.82 ± 0.05)
Lead†	8.20	7.47	8.64	N/A

* The affinity estimates were derived from [^{3}H]NMS displacement experiments and represented the mean (±SEM, n = 3–5) for the negative logarithm of K_i. The Hill coefficients are given in parentheses. To ensure that the experimental conditions are consistent, the receptor binding values of atropine were determined simultaneously with the representative soft anticholinergics at each experiment.

† Data were adapted from D'Agostino *et al.*[42]

In Vivo Pharmacodynamic Evaluation

Mydriatic Studies

First, the dose and mydriatic response relationship was established by administering increasing concentrations of the compounds until the maximum dilation was achieved. The lowest dose that produces the maximum achievable dilation was used for comparison. Accordingly, atropine (0.3% w/v), tropicamide (0.33% w/v), 13a (0.3% w/v), 13b (0.5% w/v), and 9a (0.5% w/v) produced equivalent mydriasis. Compound 9b was not studied because of its low solubility. The maximal dilation was observed within 1 h after administration without any significant differences among soft drugs, atropine, and tropicamide. In order to adequately compare the duration of the mydriatic action of soft anticholinergics with those of atropine and tropicamide, the area under the response curve in 24 h (AUC_{24h}) was calculated with trapezoidal rules for each compound in each trial. The results are shown in upper panel of Fig. 4. The AUC_{24h} of 13a was significantly smaller than that of tropicamide ($p < 0.05$). The AUC_{24h}s of 13a and 13b were significantly smaller than that of atropine sulfate ($p < 0.05$). The time courses of mydriatic activity of the treated eyes are depicted in middle panel of Fig. 4. The recovery time for atropine, tropicamide, 9a, 13a, and 13b are 24 h, 10 h, 20 h, 6.5 h, and 8.0 h, respectively.

Cardiac Studies

The cardiac effects of the soft anticholinergics were assessed by measuring the extent and duration of their bradycardia protective activities. Intravenous injection of carbachol at a dose of 5–8 μM (27–44 pmol/kg) to male Sprague-Dawley rats produced the temporary development of sinus bradycar-

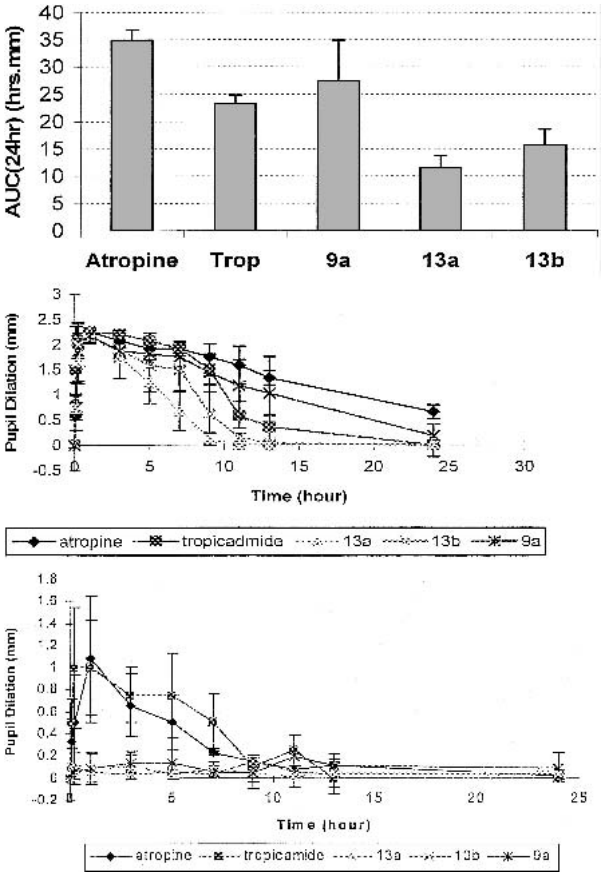

Fig. 4. Mydiatic studies of soft drugs, atropine, and tropicamide. Top panel, Comparison of the mydriatic activity of soft drug with atropine and tropicamide by AUC_{24h} method (treated eye) at equieffective doses. Middle panel, Time course of mydriatic response (treatment eye) after administration of atropine, tropicamide, 13a, 13b, and 9a. Bottom panel, Time course of mydriatic response (control eye) after administration of atropine, tropicamide, 13a, 13b, and 9a.

dia and Mobitz II A-V block, safely and reproducibly. This effect can be fully prevented by prior administration of an anticholinergic agent such as atropine or scopolamine. The bradycardia protective effects of the different anticholinergics differed greatly in terms of their extent and duration of action. In order to compare the duration of action of the soft anticholinergics and atropine, the approximate pharmacodynamic equivalent doses were administered to Sprague-Dawley rats. After the administration of two compounds, 9a (2 μM) or 13a (2 μM), the Mobitz II A-V block appeared in about 15–20 min (Fig. 5). On the other hand, atropine methyl bromide displayed protective effects against Mobitz II A-V block for at least 60 min. Actually, in preliminary experiments, duration of action longer than 2 h was found for atropine methyl bromide.

In Vitro Stability Studies

The enzymatic target of the soft drugs is the ester function, which is expected to undergo facile, one-step hydrolysis by blood and tissue esterases. The stability and metabolic pathways of the present soft drugs in rat plasma, blood, and liver homogenate were investigated. The pseudomonomolecular rates of disappearance of the drugs in the media were

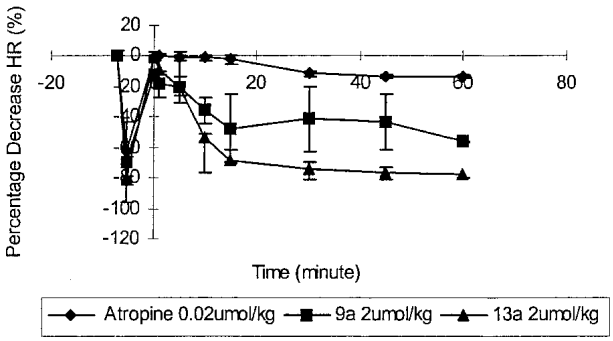

Fig. 5. Bradycardia protective effects of 9a, 13a, and atropine sulfate as illustrated by the percentage change in the heart rate.

used to calculate their $t_{1/2}$ values. As shown in Table II, although it was not significant in some cases, the methyl esters were relatively less stable than the ethyl esters. Also, there was no apparent stability relationship between the endo and exo isomers. Curiously, the hydrolysis was much faster in plasma than in blood or liver homogenate, probably indicating that erythrocyte binding of the compounds was significant. The overall results of the *in vitro* biotransformation studies of the compounds demonstrated that the "soft" quaternary ammonium acid ester linkage provided a metabolically sensitive spot that allowed the facile inactivation of molecules.

Pharmacokinetic Studies

Pharmacokinetic studies on the selected 13a were performed in rats. The concentrations of the compound in the blood samples were determined by HPLC. Because of the low detection limit (1 μg/ml) of the HPLC system, relatively high doses were used in the pharmacokinetic studies. As shown on the blood concentration curves in Fig. 6, 13a was eliminated from the blood in a biphasic manner. The data were analyzed by noncompartmental and compartmental methods, and the resulting pharmacokinetic parameters are listed in Table III. The concentration–time curves were very well described by an i.v. bolus two-compartmental model according to a biexponential equation, $C = Ae^{-\alpha t} + Be^{-\beta t}$. The statistics on the correlation coefficents of variation, >0.995, and the model selection criterion, range 3.8–5.7, indicating the goodness of fit. As Table III displays, at doses of 5, 10, and 15 mg/kg, the half-lives (15, 48, and 60 min) and elimination constants (0.23, 0.12, and 0.11 min^{-1}) showed dose dependence. The total body clearance, although similar at the doses of 15 mg/kg (11.72 ± 0.35 ml/min/kg) and 10 mg/kg (11.01 ± 0.69 ml/min/kg), was clearly higher at the dose of 5 mg/kg (16.31 ± 0.63

Table II. *In Vitro* Stability of Compounds 9a, 9b, 13a, and 13b*

Compound	Rat plasma	Rat blood	Rat liver homogenate
9a	15.21 ± 8.5	150 ± 15.97	111.68 ± 7.10
9b	21.88 ± 8.21	185.30 ± 15.92	182.29 ± 15.69
13a	30.26 ± 2.09	105.30 ± 20.66	94.61 ± 5.41
13b	34.29 ± 4.80	190.80 ± 25.32	99.89 ± 18.72

* Half-lives (min) of the compounds in biological media. Data are the mean (±SD) of three determinations.

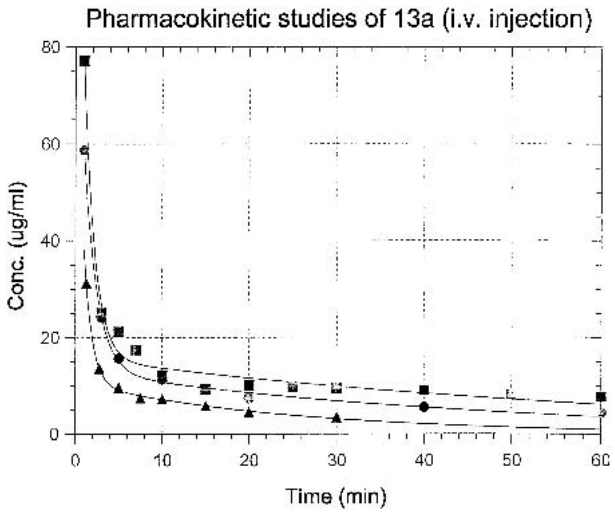

Fig. 6. Mean plasma concentration-time profiles after intravenous injection at a dose of 5 mg/kg (▲), 10 mg/kg (●), or 15 mg/kg (■).

ml/min/kg). A pharmacokinetic study was also performed at low doses such as 1 and 0.5 mg/kg. The results indicated a rapid disappearance of 13a from the blood. However, because of the detection limit in the HPLC, the data were not used for pharmacokinetic analysis.

DISCUSSION

Muscarinic receptors are involved in the control of functions of many organs in the body. Three major muscarinic receptor subtypes—M_1, M_2, and M_3—mediate a variety of basic functions of the body (27). These receptors have specific locations and control particular physiologic activities. However, most of the currently available anticholinergic agents are not subtype selective and inhibit all muscarinic subtype receptors in the body equally once they are in the systemic circulation. Thus, therapeutics for an intended symptom usually results in many undesired effects.

To improve the therapeutic index (TI) of anticholinergics, the soft drug design principles were applied to anticholinergics from the early 1980s (52). The safer anticholinergics were primarily designed for topical application, such as antiperspirants (52), mydriatics (53,54), and antiulcer drugs (21), and their facile, predictable hydrolytic deactivation assured the higher TI. With the advance of molecular biology, muscarinic subtype receptors were cloned (28,29) and became readily available. This has facilitated the drug development efforts to find anticholinergics with muscarinic receptor subtype selectivity: a few of the compounds already discovered have reached clinical trials (36). In the present study, we were trying to improve therapeutic index for anticholinergics by combining these two approaches: to develop soft anticholinergics with muscarnic receptor subtype selectivity (M_3/M_2). Soft anticholinergics with subtype selectivity (SASS) can be used not only as safer antiperspirants, mydriatics, and antiulcer agents but also as safer preoperative medications for inhibiting excessive secretion. Anticholinergics, such as atropine and glycopyrrolate, are frequently used for premedication to reduce oral and respiratory secretion and prevent bradycardia (1). They are also administered with neostigmine for the reversal of nondeploarizing neuromuscular blockade (3–5). However, such application has been complicated by the cardiac side effects of anticholinergics (2–5), particularly dangerous to the patients with preexisting cardiac diseases (40). SASS (M_3/M_2) will assist the safer administration in anesthesia, either as premedication for reducing excessive salivation and secretion of the respiratory tract induced by administration of general anesthetic agents or as an agent to reverse the nondeplorizing neromuscular blockade. Because of their muscarinic receptor subtype selectivity (M_3/M_2), the occasional serious arrhythmias associated with the conventional anticholinergics are expected to be greatly reduced when SASS are used in an anesthetic procedures, and the soft nature of these compounds will allow us to titrate the needed dose during these procedures.

Table III. Pharmacokinetic Parameters of 13a After Intravenous Bolus Administration to Rats*

Dose (mg/kg)	5 (n = 3)	10 (n = 4)	15 (n = 4)
AUC (μg · min/ml)	307.0 ± 11.9	945.0 ± 77.4	1278.5 ± 54.8
Cl_{tot} (ml/min/kg)	16.3 ± 0.6	11.0 ± 0.7	11.7 ± 0.4
MRT (min)	16.9 ± 0.9	56.9 ± 6.6	72.7 ± 12.5
V_{dss} (ml/kg)	274.6 ± 4.6	577.5 ± 54.7	896.5 ± 205.2
A (μg/ml)	60.3 ± 15.8	115.6 ± 30.6	117.3 ± 12.4
B (μg/ml)	11.5 ± 0.4	12.1 ± 1.3	12.9 ± 2.3
α (1/min)	0.95 ± 0.18	0.73 ± 0.24	0.68 ± 0.09
β (1/min)	0.045 ± 0.005	0.014 ± 0.001	0.012 ± 0.001
$t_{1/2}$ (β) (min)	14.6 ± 1.0	47.5 ± 4.2	59.5 ± 9.0
V_{dc} (ml/kg)	73.1 ± 15.6	90.2 ± 21.6	116.6 ± 13.1
V_{darea} (ml/kg)	365.5 ± 26.6	698.3 ± 69.0	1045.2 ± 167.7
k_{el} (l/min)	0.23 ± 0.04	0.12 ± 0.03	0.11 ± 0.00

* Each value represents mean ± SD of 3–4 trials.

In the mydriatic studies, it was shown that at pharmacodynamic dose-equivalent doses, two of the soft anticholinergics, 13a and 13b, displayed shorter duration than tropicamide, the most frequently used short-term mydriatic agent in the market; all soft anticholinergics tested, except 9a, showed significantly shorter duration of mydriatic action in the treated eye than atropine sulfate. In our studies, the recovery time of tropicamide was 10 h, which is different from the published data of 6 h (22,53). It might be caused by physiologic differences between the groups of rabbits. At the equieffective dose, all soft anticholinergics tested, except 9a, showed significantly shorter duration of mydriatic action in the treated eye than atropine sulfate. In the present study, the methyl esters (soft anticholinergics) displayed shorter duration than did the ethyl esters, which was inconsistent with the previous findings in the literature (55). In our studies, the lowest concentration needed to achieve the maximum pupil dilation was generally in agreement with the receptor binding data. For 9a, 13a, and 13b, the m_3 receptor binding values were almost the same (9a = 8.99, 13a = 8.49, 13b = 8.62), the lowest concentrations required to reach maximum pupil dilation were virtually the same (9a = 0.5%, 13a = 0.3%, 13b = 0.3%). For 9a, even though its binding value (m_3, pK_i = 8.99) was a little bit higher than that of 13a and 13b, it needed a slightly higher concentration to achieve the maximum pupil dilation. This could be related to its endo isomer structure, which may be unfavorable to bind to the receptor in iris-ciliary body.

The soft anticholinergics were found to cause no or mild irritation on topical administration into the eye. Reports in the literature suggested a relationship between the length of

hydrophobic side chains and irritation potential of pharmaceutical compounds (6). As the soft anticholinergics tested have generally shorter side chains (methyl and ethyl group), they may cause less irritation. The time courses of mydriatic activity in the untreated eyes (at the equieffective dose) are depicted in the bottom panel of Fig. 4. Significant dilation of the untreated eyes was observed with atropine sulfate and tropicamide, but not with the soft drugs. It suggested that the "hard drugs" atropine and tropicamide were able to dilate the untreated eye after systemic absorption. Soft drugs, however, during and after absorption into the systemic circulation, underwent a one-step deactivation to the inactive metabolites, and therefore, the systemic toxicities were expected to be significantly reduced.

In cardiac studies, compared with atropine, the shorter cardiac protective effect of equipotent soft drug doses was consistent with the facile hydrolysis of the soft anticholinergics to inactive metabolite. Juhasz *et al.* (25) reported studies on duration of antagonism of carbachol-induced brandycardia of several other types of soft anticholinergics, finding that methoxycarbonylphenylcyclopentylacetyl-N,N-dimethyltropinium methyl sulfate (PCMS-II), a different type of hindered ester, also had a duration time of 15–30 min. The present 9a and 13a, although different type of structures, possessed similar duration of action. However, tematropium sulfate, which lacked a bulky group in the moiety, had duration of cardiac protective action of only 3 min (43).

In pharmacokinetic studies, 13a displayed a saturated nonlinear pharmacokinetics. Generally, a nonlinear pharmacokinetics occurs when the drug is involved with in biologic processes, such as active membrane transport and enzyme-mediated biochemical reaction. The soft drug, by design, would be targeted to ubiquitous esterase in various organs. In general, all enzyme-mediated reactions have the capacity limitation of the saturable nature of the active sites of enzymes (56). As the higher concentration of soft drug was introduced into the systemic circulation by i.v. bolus, the hydrolyzing capacity of the esterase in the body was saturated, leading to a slower rate of elimination; thus, the nonlinear pharmacokinetic phenomenon occurred. Bodor *et al.* (57) observed dose-dependent elimination of the soft corticosteriod Loteprednol Etabonate (LE) when they studied the disposition of LE in rats. The saturable processes in the hydrolysis of the parent soft drug and the elimination of inactive metabolites via bile were believed to cause such nonlinear pharmacokinetics. In the clinical setting, either used as mydriatic or antiperspirant, anticholinergic agents are administered at a lower dose, 1–2 drops (25–50 μl) of 5 mg/ml solution in the eye (58); 0.1 ml of 5 mg/ml of solution on the skin (16). The amounts of soft drugs absorbed through eye or skin would be too low to saturate the esterase active site in the systemic circulation. Thus, the soft anticholinergics are expected to hydrolyze rapidly to the corresponding inactive metabolites after producing the desired activity, consistent with the "locally active and systemically inactive" concepts.

In conclusion, a new class of soft anticholinergics has been designed and synthesized. Receptor binding studies using cloned muscarinic receptors indicate that this new class represents potent anticholinergic agents. Two compounds of the series show muscarnic subtype receptor selectivity (M_3/M_2). Mydriatic and cardiac studies demonstrate the soft nature of the compounds. Stability and pharmacokinetic studies suggest that the new soft anticholinergics are rapidly eliminated from plasma (systemic circulation) after i.v. administration.

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