1st Department of Medicine at the University Medical School of Debrecen[1], Hungary, and Center for Drug Discovery[2], College of Pharmacy, University of Florida

Design, pharmacokinetic, and pharmacodynamic evaluation of soft anticholinergics based on tropyl α-phenylcyclopentylacetate

F. Huang[2], W.-M. Wu[2], F. Ji[2], A. Juhász[1] and N. Bodor[2]

Four new soft anticholinergic agents based on tropyl α-phenylcyclopentylacetate, **15a**, **15b**, **18a**, and **18b**, were designed and synthesized. Receptor binding studies on the cloned human muscarinic receptors indicated that the new soft anticholinergic agents possessed moderate potency as pKi ranged from 6.7 to 7.6. Mydriatic studies in rabbit eyes revealed that the duration of the action of the new soft anticholinergics (8.5 ∼ 11.0 h) were shorter than that of atropine (about 24 h) under pharmacodynamic equivalent dose, and one of them, **18a**, showed even shorter than that of tropicamide. In addition, after unilateral administration, significant dilation of pupil in the control eyes was observed with tropicamide and atropine but not with soft drugs, suggesting the systemic activity of soft drugs was minimal. With their soft nature, the new soft anticholinergics displayed much shorter protective effect against carbachol-induced bradycardia (about 30 min) than atropine (at least 60 min) in rats. *In vitro* and *in vivo* pharmacokinetic studies demonstrated that the soft anticholinergics were rapidly hydrolyzed into the corresponding inactive metabolites once they were introduced into the systemic circulation.

1. Introduction

Atropine and scopolamine analogs of *Belladonna* alkaloids have been used topically as mydriatics for a long time [1, 2]. However, such applications were complicated due to numerous unwanted systemic toxic effects and extremely long duration of action [3–5]. Successful attempts have been made in our laboratory during the past decade to design safer and short-acting mydriatic agents based on atropine and scopolamine analogs [6–10]. The inactive metabolite approach of the soft drug design concept was utilized for the design of these drugs. Briefly, a hypothetical inactive metabolite (a *Belladonna* alkaloid analog) was selected as a lead, which was then reactivated by esterifying with suitable alcohol to yield a series of active compounds. The soft drugs obtained from this approach were potent and short-acting anticholinergics with less toxic systemically after topical ocular administration. One of the obtained soft anticholinergics, tematropium methyl sulfate (the methyl sulfate of the ethyl ester of [±-α-(carboxyl)-8-methyl-8-azabicycl (3.2.1)oct-3-yl-benzene acetate]) has successfully reached Phase II human trials as a short acting mydriatic diagnostic agent.

We have recently reported design and evaluation of new soft anticholinergics: PCMS-I and PCMS-II [10]. During the process of discovery we found that some quaternary salts of tropyl α-phenylcyclopentylacetate (Scheme 1) showed very potent anticholinergic activity. For instance, α-phenylcyclopentylacetyl-*N*,*N*-dimethyltropinium methyl bromide had a PA_2 value of 8.5 [10], and a muscarinic receptor m_3 subtype binding value of 8.9 (unpublished results). Tropyl α-phenylcyclopentylacetate was easy to make (Scheme 1) ensuring the ready availability of itself and its quarternary derivatives.

Here we report design and synthesis of a group of soft anticholinergics based on tropyl α-phenylcyclopentylacetate. A hypothetical metabolite **XV** (Scheme 2) was chosen as lead metabolite for the design of the soft drugs. Even though **XV** has not been detected as the metabolite, it is a logical choice as a lead compound. Since it is the highest oxidized state of the equatorial group of N-alkyl substituent, which will ensure to "avoid the oxidation reaction during the *in vivo* metabolism as much as possible" [11]. The designed soft drugs would go through a one-step hydrolysis back to **XV** *in vivo*. The hydrolysis is expected to be facile due to the abundant presence of nonspecific esterases in ocular tissue [12] and skin [13], the possible target tissues for the soft drugs, as well as in the systemic circulation. Thus a local action without systemic side effects would be achieved with these soft drugs. An additional advantage would probably be a short duration of mydriatic action due to the increased hydrolytic lability of the soft drugs at the site of action, i.e., the iris-ciliary body of the eye. Another hypothetical metabolite **XVIII** is a positional isomer of **XV**. **XVIII** has the equatorial instead of axial position of the methyl group. It has been shown that the smaller the size at equatorial N-substituent of N-alkyl-nor-tropine esters of 2-phenyl-cyclohexenic acids, the more potent the compounds are [14]. Therefore, it is expected that the soft drug based on **XVIII** as the lead will be more potent than those based on **XV**.

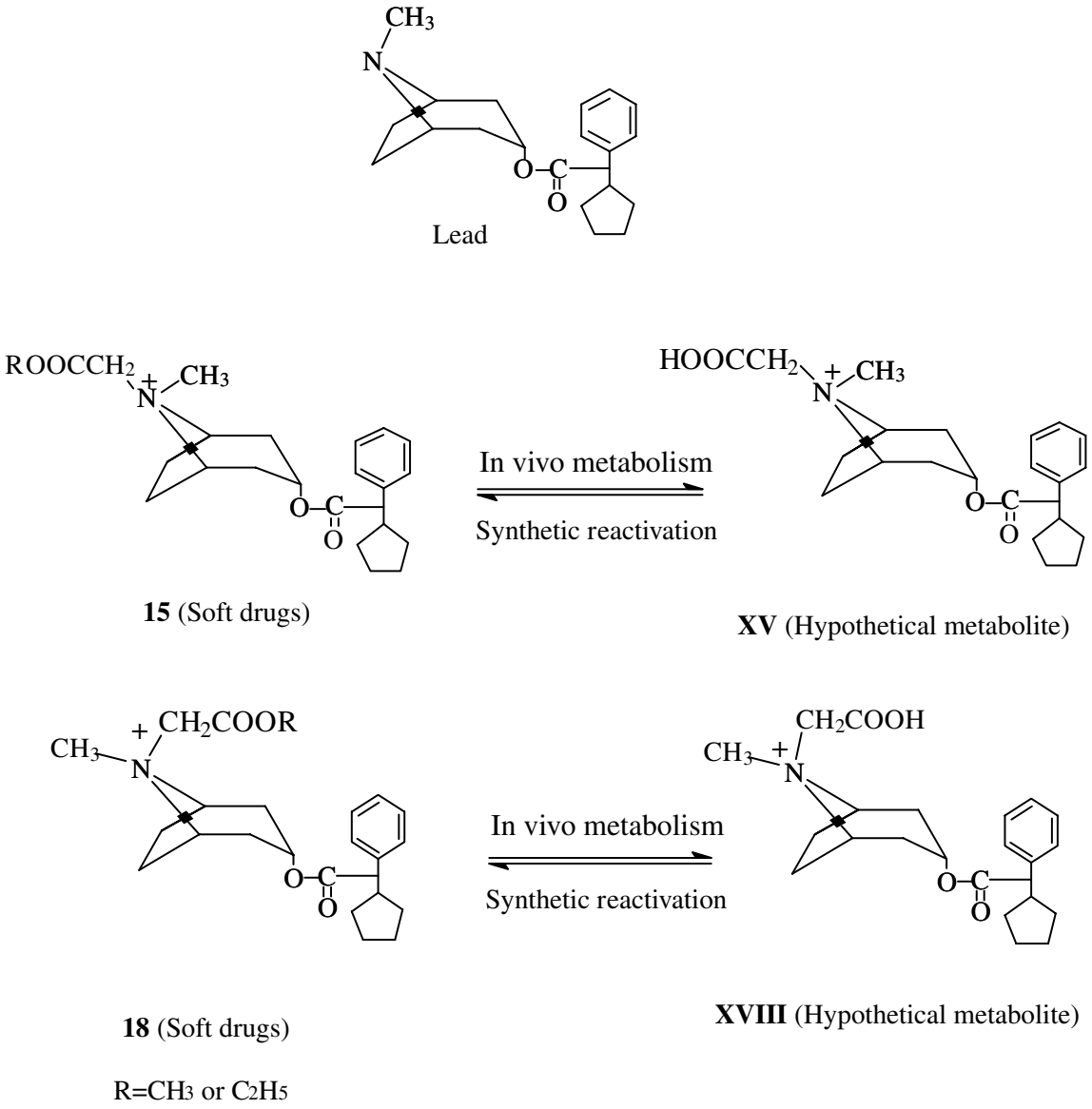
2. Investigations, results and discussion

2.1. Chemistry

Four soft anticholinergics, **15a**, **15b**, **18a**, and **18b** were designed and synthesized based on tropyl α-phenylcyclopentylacetate (Scheme 2).

Utilization of tropine HCl salt instead of tropine for the synthesis of **14** has been shown to increase the yield, because HCl is able to neutralize some of the electronic density of the lone electron pair at the nitrogen of tropine. The lone electronic pair might be interfered with the nucleophilic attack of tropine's hydroxyl group to α-phenylcyclopentaneacetyl chloride. The current method of synthesis of **14** resulted in significant increase of the yield to 78% as compared with our previously reported yield of 48% [10]. The synthesis of **15a** and **15b** were accomplished by quanternizing tropyl α-phenylcyclopentylacetate (**14**) with the corresponding bromoacetyl ester. The configuration at the ring nitrogen atom in the quaternary tropane derivatives depends on the sequence in which the individual alkyl groups are introduced [15, 16]. The alkyl group that entered last has been shown to enter the equatorial position. The syntheses of **17a** and **17b** were accom-

Scheme 1 Design of soft anticholinergies based on tropyl α-phenylcyclopeneacetate



plished by adapting the method of Koreeda and Lueng [17] through the initial demethylation of **14** then followed by the introduction of the alkoxycarbonylmethyl group. Quaternization of **17a** and **17b** with dimethyl sulfate produced the soft drugs **18a** and **18b**. The remarkably easy method of synthesis of both **15** and **18** series of soft drugs provided an universal way to design and synthesis of many soft analogs of belladonna alkaloids, such as atropine, scopolamine, and homatropine, ipratopium, andtiotropium, etc.; since all of them have a tropine moiety in their structure.

2.2. Pharmacodynamics

2.2.1. In vitro PD evaluation-receptor binding studies

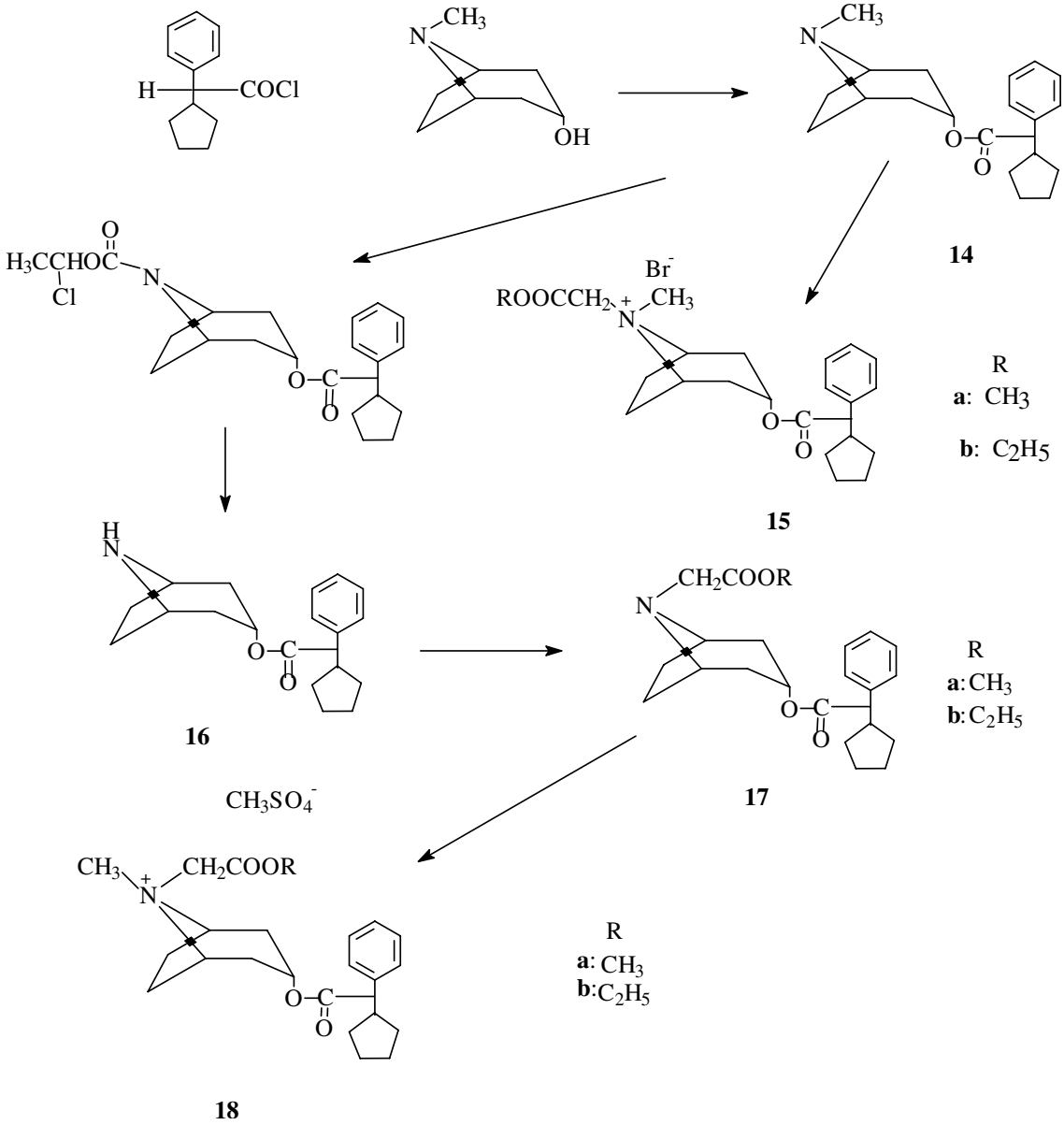
Receptor binding studies were performed on the newly synthesized soft anticholinergics based on tropyl α-phenylcyclopentylacetate. The results are listed in Table 1. The pKis of the soft drugs were in the range of $6.9 \sim 7.6$, which were about 1 magnitude less than that of atropine and lead compound. As expected, the soft drugs did not show subtype selectivity, since they were the analogs of atropine. The results showed that the pKi's of methyl derivatives (**15a** and **18a**) were higher than that of ethyl derivatives, indicating the methyl derivatives were relatively

Table 1: Receptor binding values[a] of 15a, 15b, 18a, and 18b

Compd.	m_1	m_2	m_3
15a	7.65 ± 0.01 (0.83 ± 0.04)	7.54 ± 0.18 (0.78 ± 0.02)	7.75 ± 0.10 (0.73 ± 0.01)
15b	7.42 ± 0.04 (0.87 ± 0.02)	7.20 ± 0.03 (0.83 ± 0.04)	7.57 ± 0.08 (0.75 ± 0.01)
18a	7.33 ± 0.10 (0.76 ± 0.03)	7.14 ± 0.06 (0.85 ± 0.07)	7.51 ± 0.15 (0.80 ± 0.06)
18b	7.00 ± 0.08 (0.88 ± 0.05)	6.94 ± 0.09 (0.85 ± 0.07)	7.21 ± 0.20 (1.01 ± 0.03)

[a] The affinity estimates were derived from [^{3}H]NMS displacement experiments and represented the mean ($\pm$ S.E.M, $= 3-5$) for the negative logarithm of Ki. The Hill coefficients are given in parentheses. To ensure the experimental conditions are consistent, the receptor binding values of atropine were determined simultaneously with the representative soft anticholinergics at each experiment.

Scheme 2 Synthesis of **15a**, **15b**, **18a** and **18b**



more potent than ethyl derivatives. It is in agreement with previous findings from our laboratory [10, 18] that the smaller the molecule size, the more potent the compound. The α isomers and β isomers showed similar potency. However, the β isomer showed somewhat higher affinity toward muscarinic receptors.

2.2.2. In vivo PD evaluation-mydriatic studies

Firstly, the dose-mydriatic response relationship was established by administering increasing concentrations of the compounds until the maximum dilation was achieved. The lowest dose that produced the maximum achievable dilation was considered as pharmacodynamic equivalent dose for the comparison of the duration of mydriatic activity. In this study, atropine (0.3% w/v), tropicamide (0.33% w/v), **15a** (1% w/v), **18a** (1% w/v), and **18b** (2% w/v) produced equieffectiveness. **15b** had very low solubility, therefore it was not studied. One hour after administration, the maximal dilation was observed in all rabbits treated with soft drugs, atropine, and tropicamide without significant difference between each other. Using the pharmacodynamic equivalent doses, the time course of mydriatic activity of the treated eye is shown in Fig. 1. The recovery time for atropine, tropicamide, **15a**, **18a**, and **18b** were 24 h, 10 h, 8.5 h, 11 h, and 11 h, respectively. In order to adequately compare the duration of the mydriatic action of soft anticholinergics with atropine and tropicamide, the area under response curve of 24 h ($AUC_{24\,h}$) was also calculated with trapezoidal rules for each compound of each trial. The results are presented in Fig. 2. The mydriatic durations determined by the $AUC_{24\,h}$ method were 23.49 ± 2.22, 14.3 ± 3.46, and 21.56 ± 6.8 mm · h for **15a**, **18a**, and **18b**, respectively; The corresponding AUC_{24hr} for tropicamide and atropine were 23.19 ± 1.64 and 34.92 ± 1.9 mm · h, respectively. Using both $AUC_{24\,h}$ and pupillary recovery time methods, we can conclude that the duration of mydriatic action of **18a** is shorter than that of tropicamide, the most frequently used mydriatic agent in the market. The duration of mydratic action of all soft drugs tested are shorter than that of atropine. The lowest concentration needed to achieve the maximum pupil dilation was generally in agreement with the receptor binding data. For **15a**, **18a**, and **18b**, the pKi (m_3) were 7.86,

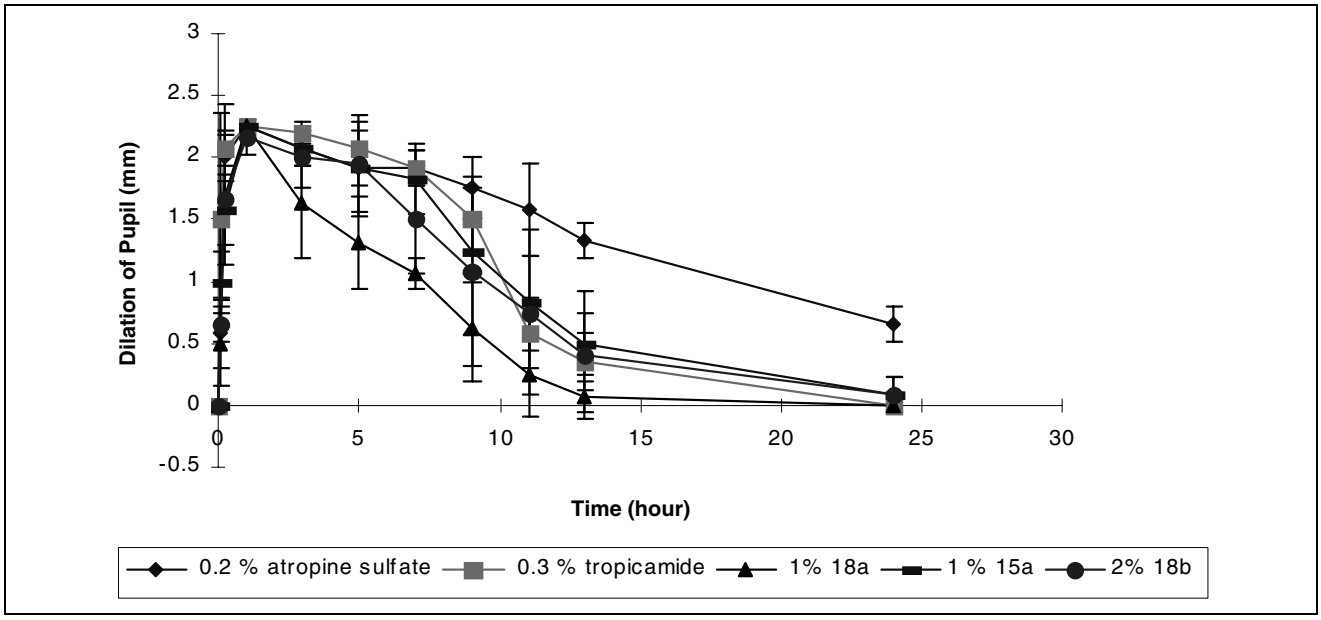


Fig. 1: Time course of mydriatic response (treated eye) after unilateral administration of atropine sulfate, tropicamide, **18a**, **15a**, and **18b**

7.23, and 6.86, respectively, and the lowest concentration needed to achieve the maximum were 1%, 1% and 2%, respectively. These results indicate that the *in vitro* and *in vivo* activities of the studied soft anticholinergics correlate very well.

The time course of mydriatic activity in the untreated eyes (at the equieffective dose) is illustrated in Fig. 3. Significant systemic absorption and subsequent control eye dilation occurred following unilateral atropine and tropicamide administration, but not with soft drugs. The lack of untreated control eye dilation in the soft drug treated rabbit may indicate systemic esterase deactivation of the soft drugs. These results support the fact that these soft drugs are nontoxic systemically.

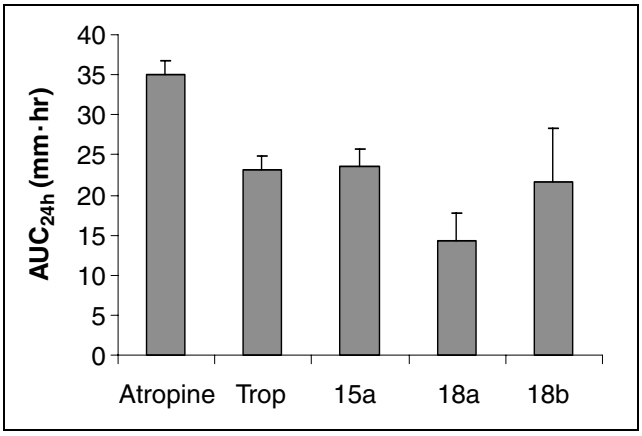


Fig. 2: $AUC_{24\,h}$ of soft anticholinergics, atropine, and tropicamide

2.2.3. In vivo PD evaluation-cardiac studies

With the intravenous administration of carbachol at a dose of 5–8 μg/kg (27–44 pmol/kg), to male Sprague-Dawley rats, the temporary development of sinus bradycardia and Mobitz II A-V block can be evoked safely and repeatedly. This effect can be antagonized (protected) by the previous administration of an anticholinergic agent, e.g., atropine, scopolamine, or soft anticholinergics. In order to compare the duration of the soft anticholinergics and atropine, the approximate pharmacodynamic equivalent dose based on the receptor binding value (m_2) was i.v. administered into Sprague-Dawley rats. In this study, **15a** (0.2 μmol/kg and 2 μmol/kg) and atropine (0.02 μmol/kg) were tested and compared. As displayed in Fig. 4, the protective effect of **15a** was about 30 min (the time for the appearance of Mobitz II A–V block). However, atropine displayed such protective effect for at least 60 min; actually, in preliminary experiments, a duration of protective action longer

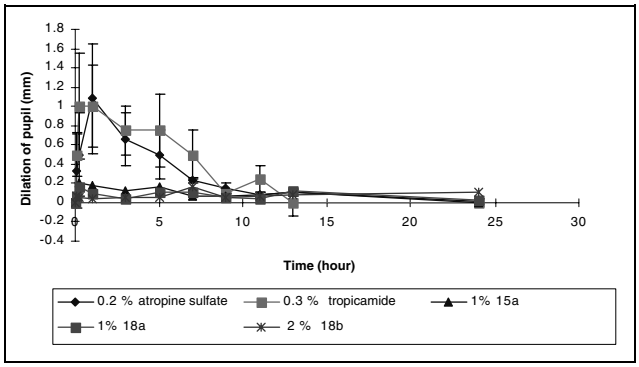


Fig. 3: Time course of mydriatic response (control eye) after unilateral administration of atropine sulfate, tropicamide, **15a**, **18a**, and **18b**

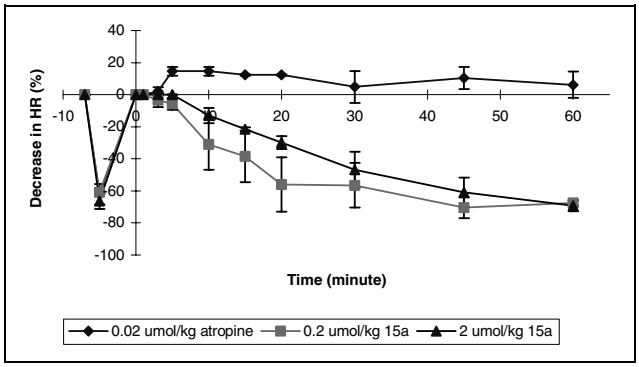


Fig. 4: Bradycardia protective effect of **15a** and atropine MeBr as illustrated by percentage heart rate change.

than 2 h was found for atropine methyl bromide (atropine MeBr). The relatively shorter duration of cardiac effect of soft drug **15a** reinforced the fact that the soft anticholinergic was rapidly hydrolyzed into the inactive moiety once it entered the systemic circulation.

2.3. Pharmacokinetics

2.3.1. In vitro PK-biotransformation studies

To prove that the soft drugs possess appropriate properties for the clinical application, the stability and metabolic pathways in blood and liver homogenate obtained from rats were investigated. The rates of disappearance of the drugs were used to calculate their $t_{1/2}$ values. In the biological media, the metabolism of the soft anticholinergics appeared to be a pseudo first-order kinetic, and all soft drugs converted to the acid metabolites. As shown in Table 2, the methyl ester derivatives were more rapidly hydrolyzed than the ethyl ester derivatives; and the rate of hydrolysis of α-isomers were similar to that of β isomers. These results indicate the general trend that the longer and the more steric hindered the side chain, the longer the hydrolytic half life of the compond [7, 19]. The hydrolytic rates of soft drug in rat plasma were about 20 to 30 min, however, the hydrolytic rates of soft drug in whole blood were in the range of 180 to 300 min, about 10 times longer than in plasma, suggesting that erythrocyte binding of the compounds was significant. These results are similar to the *in vitro* biotransformation studies of soft drug based on propantheline [20]. The overall results of the *in vitro* biotransformation studies of the compounds demonstrate that the "soft" acyloxyalkyl linkage provides a metabolically sensitive spot, which allows the facile decomposition of the soft analogs to the inactive moieties.

Table 2: *In vitro* stability of 15a, 15b, 18a, and 18b[a]

Compd.	at plasma	at blood	at liver homogenate (20%)
15a	23.64 (8.32)	171.35 (34.81)	186.60 (68.14)
15b	38.27 (7.15)	285.92 (42.22)	274.89 (82.23)
18a	17.48 (10.4)	185.85 (32.29)	179.24 (94.34)
18b	29.04 (12.12)	301.05 (60.15)	275.98 (101.79)

a: Half-lives (min) of the compounds in biological media. Data are the mean (SD) of two to three determinations.

Table 3: Pharmacokinetics of 15a after intravenous administration of 10 mg/kg

Parameters	Rat 1	Rat 2	Rat 3	Mean (SD)
AUC, μg · min/ml	234	247	222.28	234.42 (12.36)
AUMC, μg · min^2/ml	5611	5150	4641	5125.67 (497.94)
MRT, min	23.97	20.85	20.76	21.86 (1.83)
Cl_{tot}, ml/min/kg	42.73	40.48	44.98	42.73 (2.25)
V_{dss}, ml/kg	1024.73	844.13	934.25	934.37 (90.29)
$t_{1/2}$, min	15	42	38	31.67 (14.57)
Fe, %[a]	12.4	10.3	8.3	10.3 (2.05)

a: percentage of dose excreted as unchanged drugs.

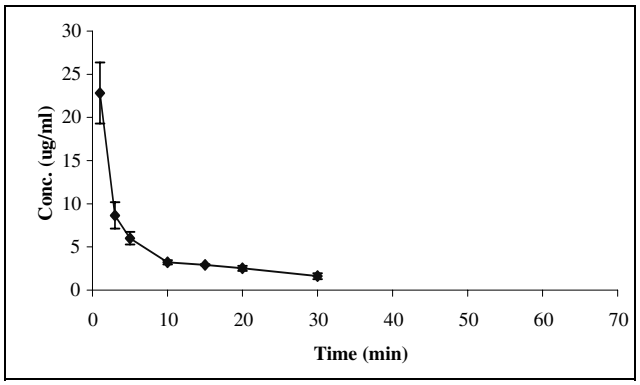


Fig. 5: Mean plasma concentration profile of **15a** after 10mg/kg intravenous injection in rats

2.3.2. In vivo pharmacokinetic studies

The mean concentration plasma profile of **15a** after intravenous administration at a dose of 10 mg/kg is presented in Fig. 5. As shown in the graph, the **15a** plasma concentration declined in a bi-exponential manner with a fairly rapid distribution phase and short terminal half-life. The pharmacokinetic parameters of **15a** following intravenous administration of 10 mg/ml are summarized in Table 3. The terminal half-life of **15a** was about 30 min, indicating a relatively rapid elimination of **15a** from the systemic circulation. The mean steady-state volume of distribution was 934.37 ml/kg, which was higher than the extracellular water volume in the rat, 700 ml/kg [21], suggesting that the majority (75%) of **15a** was confined into the extracellular water compartment, and a small portion (about 25%) was intracellularly distributed. This is in agreement with the fact that **15a** is positively charged, which prevents itself from readily passing through cellular membranes. The mean total clearance of **15a** was 42.73 ml/min/kg. It has been reported that the clearance of scopolamine and atropine in rats were 74.0 ml/min/kg [22] and 58.0 ml/min/kg [23], respectively, however, the analytical method for the determination of the plasma concentration for both scopolamine and atropine were radioreceptor assay. Kentala and Thiermann pointed out that radioassay measures only the biologically active *l*-hyoscyamine, resulting in a three times higher total clearance (Cl_{tot}), as compared with the methods which measure both *l*-and *d*-hyoscyamine [24–26]. In this sense, the clearance of **15a** was higher than that of atropine and scoplamine, since the HPLC method measured both *l* and *d* enantiomers. Anticholinergic agents such as atropine and scopolamine are eliminated predominately by the renal pathway [27]. In this study, after **15a** administration, we observed a significant portion of the metabolite in urine, too. The higher total clearance of **15a** did suggest that metabolic clearance from plasma contributed significantly to the total clearance. There were about 10 to 15% of the soft drug eliminated from the urine as unchanged drug. This was most likely due to the fact that a large amount of the soft drug was (**15a**) injected into the systemic circulation as a bolus, and the active sites of the esterase enzyme were saturated by a relatively large quantity of ester functionality. Therefore, a portion of the soft drug was renally eliminated unchanged before it was hydrolyzed by the plasma esterase enzyme. In addition, the possible significant erythrocyte binding of the compounds (as suggested by the *in vitro* stability study) may prevent hydrolysis of the soft drugs *in vivo,* since only the unbound fraction of the drugs were subjected to enzymatic action. However, the bound drug is pharmacodynamicly inactive, a factor which may partly explain that the soft drugs were less active systemically during mydriatic and cardiac studies.

In conclusion, new soft anticholinergics based on tropyl α-phenylcyclopentylacetate were designed and synthe-

sized. Receptor binding studies showed that the newly synthesized soft anticholergics displayed moderate potency as compared with classical anticholinergic agents, scopolamine or atropine. The pharmacodynamic and pharmacokinetic data indicate that these anticholinergic agents were degraded into predicted inactive metabolites after entering the systemic circulation. The method to design and synthesize this new series of compounds may be used for the design and synthesis of other belladonna alkaloid agent.

3. Experimental

3.1. Materials and methods

All chemicals used were reagent grade. N-[^{3}H]-Methyl-scopolamine (NMS) was obtained from Dupont NEN Research (Boston, MA). Atropine, scopolamine, and propantheline were from Sigma Chemicals Co. (St. Louis, MO). All other chemicals were from Aldrich Chemical Company (Milwaukee, Wisconsin). Scintiverse BD and other solvents were from Fisher Scientific Co. (Pittsburgh, PA). Tropicamide ophthalmic solution (1%) was purchased from Schein Pharmaceutical Inc. (Florham Park, NJ). All m.p.'s were recorded by a Fisher-Johns apparatus and are uncorrected. NMR data were recorded with a Varian 300 NMR spectrometer and are reported in parts per million (δ) relative to tetramethylsilane. All compounds were dissolved in $CDCl_3$. The elemental analysis was carried out at Atlantic Microlab Inc. (Atlanta, GA). All the results were in an acceptable range. The was carried out using EM Science DC-Plastic foil plate coated to a thickness of 0.2 mm with silica gel 60 containing florescent (254) indicator. CC was performed with silica gel (70–230) with 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). The following strains of animals were used in the studies: (1) male New Zealand albino rabbit weight 3 kg, and (2) male Sprague Dawley rats weighing 250–300 g.

3.2. Synthesis

3.2.1. Tropyl α-phenylcyclopentylacetate **(14)**

To α-phenylcyclopentaneacetic acid (2 g, 9.79 mmol) in 15 ml of absolute ethyl ether, *N,N*-dimethylformamide (1 drop) and thionyl chloride (1.28 g, 10.77 mmol) were added at room temperature. The mixture was refluxed for 2 h. Then the ethyl ether was removed under reduced pressure to give oily α-phenylcyclopentaneacetyl chloride.

To tropine (10.77 mmol, 1.913 g) in 15 ml of nitromethane, was added the above oily α-phenylcyclopentaneacetyl chloride in 5 ml of nitromethane. Then the mixture was refluxed for 24 h. Removal of nitromethane gave an oily substance, which was basified with $NaHCO_3$. The mixture was extracted 3 times with ethyl ether to give crude tropyl α-phenylcyclopentylacetate, which was further purified by flash chromatography on silica gel (Methanol : NH_4OH = 100 : 2.5) to give pure tropyl α-phenylcyclopentylacetate (**14**, 2.5 g, 78%). ^{1}H NMR ($CDCl_3$): 0.9–2.1 [16 H, 4 × m, (CH_2CH_2)$_2$ CH and tropyl's 2,4,6,7-H], 2.23(3 H, s, CH_3 N), 2.5–2.64 [1 H, m, (CH_2CH_2)$_2$C*H*], 2.90, 3.15(2 H, br d, tropyl's 1,5-H), 3.25 (1 H, d, PhC*H*), 4.96 (1 H, t, CO_2CH), 7.29–7.36 (5 H, m, Ph) ppm.

3.2.2. Phenylcyclopentylacetyl-N$_\beta$-methoxycarbonylmethyltropinium bromide **(15a)** *and phenylcyclopentylacetyl-N$_\beta$-ethoxycarbonylmethyltropinium bromide* **(15b)**

To 2 g (6.10 mmol) of tropyl α-phenylcyclopentaneacetate (**14**) in 20 ml of anhydrous acetonitrile, 15.26 mmol of methyl bromoacetate or ethyl bromoacetate was added. The above mixture was stirred under argon for 19 h. Evaporation of acetonitrile generated an oily substance, which was further purified by precipitation (methylene chloride/ethyl ether) to give pure **15a** or **15b**.

15a (2.2 g, 80%). M.p.178–179 °C. ^{1}H NMR ($CDCl_3$): 1.00, 1.20, 1.40–1.82, 1.90–2.37 [16 H, m, (CH_2CH_2)$_2$CH and tropyl's 2,4,6,7-H], 2.71, 2.78 [1 H, 2 × br., (CH_2CH_2)$_2$C*H*], 3.24 (1 H, d, PhC*H*), 3.60 (3 H, s, NCH_3), 3.78 (3 H, s, CO_2CH_3), 4.62, 4.84 (2 H, 2 × br., tropyl's 1,5-H), 4.80, 4.92 (2 H, 2d, CH_2CO_2), 5.12 (1 H, t, CO_2CH), 7.24–7.30 (5 H, m, Ar–H) ppm.

$C_{24}H_{34}O_4NBr$

15b (2.3 g, 82%). M.p. 181–182 °C. ^{1}H NMR ($CDCl_3$): 1.00, 1.20, 1.40–1.82, 1.90–2.37 [16 H, m, (CH_2CH_2)$_2$ CH and tropyl's 2,4,6,7-H], 1.28 (3 H, t, CH_3CH_2), 2.71, 2.78 [1 H, 2 × br., (CH_2CH_2)$_2$C*H*], 3.24 (1 H, d, PhC*H*), 3.60 (3 H, s, NCH_3), 4.22 (3 H, q, $CH_3CH_2CO_2$), 4.62, 4.84 (2 H, 2 × br., tropyl's 1,5-H), 4.7, 4.82 (2 H, 2d, CO_2CH_2N), 5.12 (1 H, t, CO_2CH), 7.24–7.30 (5 H, m, Ph) ppm.

$C_{25}H_{36}O_4NBr$

3.2.3. Nortropyl α-phenylcyclopentylacetate **(16)**

To 1 g (3.05 mmol) of tropyl α-phenylcyclopentaneacetate in 10 ml of 1,2-dichloroethane at 0 °C, 1.09 g (7.63 mmole) of 1-choroethyl chloroformate in 5 ml of 1,2-dichloroethan was added dropwise. The mixture then refluxed for 1 h. Evaporation of the reaction mixture in vaco gave an oily residue, which was refluxed in methanol for 45 min. Removal of methanol under reduced pressure gave **16** (0.95 g, 99%). ^{1}H NMR ($CDCl_3$): 0.9–2.1 [16 H, 3 × m, (CH_2CH_2)$_2$CH and tropyl's 2,4,6,7-H], 2.51–2.53 [1 H, 2br, (CH_2CH_2)$_2$C*H*], 3.25 (1 H, d, PhC*H*), 3.80, 3.95(2 H, br d, tropyl's 1,5-H), 5.12 (1 H, t, CO_2CH), 7.29–7.36 (5 H, m, Ph) ppm.

3.2.4. Methoxycarbonylmethylnortropyl-N$_\alpha$-phenylcyclopentaneacetate **(17a)** *and ethoxycarbonylmethylnortropyl-N$_\alpha$-phenylcyclopentaneacetate* **(17b)**

To well stirred compound **16** (1 g, 3.19 mmol) in 20 ml of *N,N*-dimethylformamide (DMF) with K_2CO_3 (1.0 g), was added 3.19 mmol of methyl bromoacetate or ethyl bromoacetate. The mixture was stirred under argon for 20 h. Then the DMF was removed under reduced pressure. The residue was extracted 3 times with ethyl ether to give 1.12 g of crude **17**, which was further purified by flash chromatography on silica gel with ethyl acetate to give pure **17a** (0.98 g, 79.7%) or **17b** (1.01g, 79.2%).

17a (0.98 g, 79.7%). ^{1}H NMR (CDCl3): 0.9–2.1 [16 H, 5 × m, (CH_2CH_2)$_2$CH and tropyl's 2,4,6,7-H], 2.5–2.7 [1 H, m, (CH_2CH_2)$_2$C*H*], 3.1, 3.2 (2 H, brd, tropyl's 1,5-H), 3.15 (2 H, s, NCH_2), 3.23 (1 H, d, PhC*H*), 3.70 (3 H, s, OCH_3), 4.95 (1 H, m, CO_2CH), 7.20–7.40 (5 H, m, Ph) ppm.

17b (1.01 g, 79.2%). ^{1}H NMR ($CDCl_3$): 0.9–2.1 [16 H, 5 × m, (CH_2CH_2)$_2$CH and tropyl's 2,4,6,7-H], 1.25 (3 H, s, CH_3), 2.5–2.7 [1 H, m, (CH_2CH_2)$_2$C*H*], 3.1–3.2 (2 H, br d, tropyl's 1,5-H), 3.15 (2 H, s, NCH_2), 3.23 (1 H, d, PhC*H*), 4.18 (2 H, q, OCH_2CH_3), 5.01 (1 H, m, OCH), 7.20–7.40 (5 H, m, Ph) ppm.

3.2.5. Phenylcyclopentylacetyl-N$_\alpha$-methoxycarbonylmethyltropium methylsulfate **(18a)** *and phenylcyclopentylacetyl-N$_\alpha$-ethoxycarbonylmethyltropium methylsulfate* **(18b)**

To compound **17** (**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 dropwise adding methylene-dissloved mixture into ethyl ether to give as precipitate **18a** or **18b**.

18a (1.13 g, 88.3%). M.P. 159–160.5 °C. ^{1}H NMR ($CDCl_3$): 0.9–2.1 [16 H, 5 × m, (CH_2CH_2)$_2$CH and tropyl's 2,4,6,7-H], 2.5–2.7 [1 H, m, (CH_2CH_2)$_2$C*H*], 3.21 (3 H, s, NCH_3), 3.23 (1 H, d, PhC*H*), 3.64 (3 H, s, CH_3SO_4), 3.80 (3 H, s, OCH_3), 4.25–4.57 (2 H, br d, tropyl's 1,5-H), 4.81 (2 H, brd, NCH_2), 5.15 (1 H, t, CO_2CH), 7.20–7.40 (5 H, m, Ph).

$C_{25}H_{37}O_8NS$

18b (1.17g, 89.0%). M.p. 153–154 °C. ^{1}H NMR ($CDCl_3$): 0.9–2.1 [16 H, 5 × m, (CH_2CH_2)$_2$ CH and tropyl's 2,4,6,7-H], 1.20 (3 H, s, CH_3), 2.5–2.7 [1 H, m, (CH_2CH_2)2C*H*], 3.19 (3 H, s, NCH_3), 3.23 (1 H, d, PhC*H*), 3.70 (3 H, s, CH_3SO_4) , 4.22 (2 H, q, O**CH_2**CH_3), 4.40, 4.53 (2 H, br d, tropyl's 1,5-H), 4.65 (2 H, m, NCH_2), 5.15 (1 H, t, CO_2CH), 7.20–7.40 (5 H, m, Ph).

$C_{26}H_{39}O_8NS$

3.3. Pharmacodynamic evaluation

3.3.1. In vitro pharmacodynamic evaluation-receptor binding studies

Binding studies were performed with [^{3}H]-methyl-scopolamine following the protocol from RBI Co. (Natick, Massachusetts). Binding buffer (Phosphate Buffered Saline-PBS, pH 7.4) consisted of 0.15 M NaCl, 1.5 mM KH_2PO_4 and 2.7 mM Na_2HPO_4. 10 mM NaF was added into the buffer as an esterase inhibitor. The assay mixture (1 ml) contained 100 µl diluted membranes (receptor proteins, final concentration: m_1, 25 µg/ml; m_2, 42 µg/ml; m_3, 15.9 µg/ml; m_4, 20 µg/ml). The final concentrations of NMS for the m_2–m_4 binding studies were 0.5 nM, and for m_1 was 1nM. Specific binding was defined as the difference in [^{3}H] NMS binding in the absence and presence of 1 µM atropine. Incubation was carried out at room temperature for 60 min. The assay was terminated by filtration through a Whatman GF/B filter (presoaked with 0.5% polyethyleneimine). The filter was then washed three times with 10 ml ice cold binding buffer, transferred to vials and 10 ml of scintiverse liquid were added. Finally, detection was performed on a Packard 31800 liquid scintillation analyzer (Packard Instrument Inc., Downer Grove, IL).

Data from the binding experiment were fitted to the following equation:

$$\%\ [3H]\ NMS.\ bound = \frac{100 \cdot x^n}{k + x^n} \quad (1)$$

to obtain Hill coefficient n.

Then fitted to

$$\%\ [3H]\ NMS.\ bound = \frac{100 \cdot IC_{50}}{k + IC_{50}} \quad (2)$$

to obtain IC_{50}, where x = concentration of the tested compound (in a series concentration). K_i was derived by the method of Cheng and Prusoff [28]: $K_i = IC_{50}/(1 + L/K_D)$, where L is the concentration of the radioligand, IC_{50} is the concentration of drug causing 50% inhibition of specific radioligand binding and K_D is the dissociation constant of radioligand receptor complex. Data were analyzed by a nonlinear least-squares curve fitting procedure using the program Scientist (MicroMath Inc., Salt Lake City, UT).

3.3.2. In vivo pharmacodynamic evaluation-mydriatic studies

Six healthy male New-Zealand White rabbits, each weighing about 3.0 kg, were used in the experiment. Drug at various concentrations in distilled water were administered to one eye while the other eye served as control. Using atropine-MeBr and tropicamide as reference compounds, the mydriatic activities of the newly synthesized soft anticholinergics were evaluated. At appropriate time intervals, pupil diameters of both treated eyes and untreated were determined for all drugs. The difference in pupil diameter between each point and zero time point were calculated for both treated and control eyes and reported as mydriatic response.

3.3.3. In vivo pharmacodynamic evaluation-cardiac studies

Male Sprague-Dawley rats (Harlan Sprague Dawley Inc. Indianapolis, IN), each weighing 300 ± 30 g, were anesthetized with of 30 mg/kg i.p. Na pentobarbital. Baseline electrocardiography (ECG) recordings and all drug administrations were performed after 15 min stabilization periods. 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). Standard leads II, III, and I were recorded at a paper speed of 25 mm/s. Recordings were taken before, during, and after the administration of any of the compounds until all basic ECG parameters returned to that of the baseline. ECG recordings were evaluated for the following parameters: PP cycle length (ms), RR cycle length (ms), heart rate (1/min) by the equation of 60000/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 analyzing the ECG recordings, the percent changes of heart rate, as compared to that of the baseline, were plotted against time, and the effects of the soft drugs and atropine on the percent changes of the heart rate were characterized. All drugs were dissolved in 0.9% NaCl (vehicle), and solutions were administered by direct injections into the jugular veins on either side of the rats. Soft anticholinergics (0.2 μmol/kg and 2 μmol/kg, in ~0.3 ml volume) or atropine (0.02 μmol/kg, in ~0.3 ml volume) were administered at 0 time, while carbachol (5–8 μg/kg = 27–44 pmol/kg in ~0.06–0.1 ml volume according to the initial individual ECG response of each rat) was injected at −5, 1, 3, 5, 10, 15, 20, 30, 45, and 60 min. Analysis of variance followed by Duncan's test was used for statistical evaluation.

3.4. Pharmacokinetic evaluation

3.4.1. In vitro pharmacokinetic evaluation – in vitro biotransformation studies

Freshly collected rat plasma, rat blood, and 25% rat liver homogenate were used. To biological medium (2 ml), 10 μl of the stock solution of the compound was added and mixed. The mixture was kept at 37 °C while being shaken. Samples (100 μl) were withdrawn at appropriate time intervals and immediately mixed with 200 μl of ice-cold 5% DMSO in acetonitrile solution to stop enzymatic reaction and then vortexed. After centrifugation, the supernatant was analyzed by HPLC for both the original compound and the metabolite.

Concentrations of soft drugs in plasma, urine, and bile were determined by reverse-phase HPLC using a Superlcosil LC ABZ column (Supelco, Bellofonte, PA). The HPLC system consisted of a Spectra Physics (San Jose, CA) SP 8810 isocratic pump, SP 8450 UV/Vis detector with detection set to 254 nm, and an SP 4290 integrator. The mobile phase (flow rate of 1.5 ml/min) consisted of acetonitrile/water (40 : 60), containing 0.1% octanesulfonic acid, 0.2% acetic acid, and 0.1% tetrahydrofuran. Retention times for **15a**, **15b**, **18a**, **18b**, **XV**, and **XVIII** were, 10.43, 14.88, 11.71, 15.30, 4.72, and 4.78 min, respectively. The assay was linear over the range of 1 μg/ml to 100 μg/ml, with a detection limit of 1 μg/ml. The intra- and inter-day coefficients of variation of the assay over the range of standard concentrations were less than 10%, and the accuracy of the assay was greater than 95%. The analytical recovery of soft drugs was greater than 97%.

3.4.2. In vivo pharmacokinetic evaluation

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg). Soft anticholinergics were injected into tail vein, over 1 min, at a dose of 10 mg /kg and a dosing volume of 1 ml/kg. The tail vein injections were conducted very carefully to assure that no leakage occurred during the injection. Blood samples, 0.1 ml, were collected through the jugular vein at appropriate time intervals for 90 min. The urine samples were taken at 2, 4, and 6 h, and the volumes of the urine were measured. The animals were sacrificed by an overdose of pentobarbital after the experiments.

Noncompartmental analysis method was applied to the analysis of pharmacokinetic results; Winnnonlin program (Pharsight Corp., Cary, NC) was used to assist PK analysis. The area under the curve, AUC, of the blood concentration versus time was calculated using the trapezoidal rule, and 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"; 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. The terminal half-life, $t_{1/2}$, was calculated as $\ln 2/\lambda z$, where λz is the elimination constant, which was determined by the slope of terminal points.

This research paper was presented during the 3rd Conference on Retrometabolism Based Drug Design and Targeting, May 13–16, 2001, Amelia Island. Florida, USA.

References

1 Brown, J. H.; Taylor, P.; in: Goodman, L. S., Gilman, A., Hardman, J. G., Gilman, A. G., Limbird, L. E. (Eds.): Goodman & Gilman's the pharmacological basis of therapeutics, p. 141, McGraw-Hill, New York 1996
2 Katzung, B.; in: Katzung, B. (Ed.) Basic & clinical pharmacology, p. 105, Appleton & Lange, Stamford 1998
3 Diamond, J. P.: Drugs Aging **11**, 352 (1997)
4 Fraunfelder, F. T.; in: Drug Induced Ocular Side Effects & Drug Interactions, 3, p. 445, Lea & Febiger, New York 1989
5 Wright, B. D.: Br. J. Ophthamol. **76**, 179 (1992)
6 Hammer, R. H.; Amin, K.; Gunes, Z. E.; Brouillette, G.; Bodor, N.: Drug Des. Deliv. **2**, 207 (1988)
7 Hammer, R. H.; Wu, W.-M.; Sastry, J. S.; Bodor, N.: Curr. Eye Res. **10**, 565 (1991)
8 Kumar, G. N.; Hammer, R. H.; Bodor, N. S.: Drug Des. Discov. **10**, 11 (1993)
9 Kumar, G. N.; Hammer, R. H.; Bodor, N. S.: Drug Des. Discov. **10**, 1 (1993)
10 Juhasz, A.; Huang, F.; Ji, F.; Buchwald, P.; Wu, W. M.; Bodor, N.: Drug Dev. Res. **43**, 117 (1998)
11 Bodor, N.: Pharmazie **52**, 491 (1997)
12 Lee, V. H.: J. Pharm. Sci. **72**, 239 (1983)
13 Tauber, U.; Rost, K. L.; in: Shroot, B., Schaefer, H. (Eds.): Skin pharmacokinetics, p. 170, Karger, Basel ; New York 1987
14 Turbanti, L.; Cerbai, G.; Garzelli, G.; Renzetti, A. R.; Criscuoli, M.; Subissi, A.; Bramanti, G.; Martinelli, A.: Eur. J. Med. Chem. **27**, 449 (1992)
15 Key, J. B.; Robinson, J. B.; Thomas, J.: J. Chem. Soc. 5112 (1965)
16 Findlay, S. P.: J. Amer. Chem. Soc. **75**, 3024 (1953)
17 Koreeda, M.; Luengo, J. I.: J. Org. Chem. **49**, 2081 (1984)
18 Kumar, G.; Huang, M. J.; Hammer, R.; Bodor, N.: J. Pharm. Sci. **83**, 117 (1994)
19 Kumar, N. Design and evaluation of soft anticholinergics based on methylscopolamine; University of Florida 1992
20 Brouillette, G.; Kawamura, M.; Kumar, G. N.; Bodor, N.: J. Pharm. Sci. **85**, 619 (1996)
21 Gerlowski, L. E.; Jain, R. K.: J Pharm. Sci. **72**, 1103 (1983)
22 Nakashima, E.; Ishizaki, J.; Takeda, M.; Matsushita, R.; Yokogawa, K.; Ichimura, F.: Biopharm Drug Dispos **14**, 673 (1993)
23 Urso, R.; Segre, G.; Bianchi, E.; Bruni, G.; Dal-Pra, P.; FIaschi, A.: Eur. J. Drug Metab. Pharmacokinet. Spec, 111 (1991)
24 Kentala, E.; Kaila, T.; Kanto, J.: Pharmacol. Toxicol. **65**, 110 (1989)
25 Kentala, E.; Kaila, T.; Iisalo, E.; Kanto, J.: Int. J. Clin. Pharmacol. Ther. Toxicol. **28**, 399 (1990)
26 Thiermann, H.; Radtke, M.; Spohrer, U.; Klimmek, R.; Eyer, P.: Arch. Toxicol. **70**, 293 (1996)
27 Ali-Melkkila, T.; Kanto, J.; Iisalo, E.: Acta. Anaesthesiol. Scand. **37**, 633 (1993)
28 Cheng, Y-C; Prusoff, W. H.: Biochem. Pharmacol. **22**, 3099 (1973)

Nicholas Bodor
Center for Drug Discovery
College of Pharmacy
University of Florida
P.O. Box 100497
Gainesville, FL 32610-0497
USA