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Full-length article

Pharmacological profiles of an anticholinergic agent, phencynonate hydrochloride, and its optical isomers[1]

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Key words

optical isomers; muscarinic acetylcholinic receptors; pharmacological profiles; radioligand binding assay

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Abstract

Aim: To comparatively study the pharmacological profiles of 3-methyl-3-azabicyclo (3,3,1)nonanyl-9-α-yl-α-cyclopentyl-α-phenyl-α-glycolate (phencynonate hydrochloride, CPG), an anticholinergic agent, and its enantiomers [*R*(–)- and *S*(+)-CPG]. **Methods:** The affinity and relative efficacy were tested using radioligand-binding assay with muscarinic acetylcholine receptors from rat cerebral cortex. The pharmacological activities were assessed in three individual experiments: (1) potentiating the effect of subthreshold hypnotic dose of sodium pentobarbital; (2) inhibiting oxotremorine-induced salivation; and (3) inhibiting the contractile response to carbachol. **Results:** The order of potency of phencynonate hydrochloride and its optical isomers to inhibit the binding of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) was *R*(–)-CPG (K_i=46.49±1.27 nmol/L)>CPG (K_i=271.37±72.30 nmol/L)>*S*(+)-CPG (K_i=1263.12±131.64 nmol/L). The results showed that *R*(–)-CPG had the highest affinity to central muscarinic receptors among the three compounds, but did not show any central depressant effects at dose from 10.00 to 29.15 mg/kg. CPG increased the effects of subthreshold hypnotic dose of sodium pentobarbital induced-sleeping [the ED_{50}±95% LC value was 21.06±3.04 mg/kg]. CPG and *R*(–)-CPG displayed nearly equipotent effect in depressing oxotremorine-induced salivation [the ED_{50} ±95% LC for *R*(–) and CPG were 1.10±0.28 and 1.07±0.15 mg/kg, respectively], and the contractile response to carbachol (pA_2 values for *R*(–) and CPG were 6.84 and 6.80, respectively). *S*(+)-CPG presented the lowest anticholinergic profiles, but could potentate effects of its enantiomers in some manner. **Conclusions:** These data suggested that *R*(–)-CPG acted as an eutomer in racemate and a competitive antagonist to acetylcholine muscarinic receptors, but *S*(+)-CPG was less active in comparison to *R*(–)-CPG and its racemate. The central depressant effects of *R*(–)-CPG and *S*(+)-CPG were lower in comparison to its racemate.

Introduction

Molecular handedness is a crucial structural feature of biologically active compounds, since opposite configurations at pharmacophoric groups frequently influence the biological response, mainly in terms of affinity, toxicity, and receptor subtype selectivity[1]. Therefore, the stereoisomeric composition of drugs is currently receiving considerable attention owing to its pharmacological as well as industrial and regulatory implications[2,3].

3-Methyl-3-azabicyclo(3,3,1)nonanyl-9-α-yl-α-cyclopentyl-α-phenyl-α-glycolate (phencynonate hydro-chloride, CPG) is a central anticholinergic agent synthesized at the Beijing Institute of Pharmacology and Toxicology. It has been developed as a new medicine for motion sickness. Our previous studies revealed that CPG prevented motion sickness with higher efficacy and lower central inhibitory side effects compared to other motion sickness drugs, such as dimenhydrinate and scopolamine HBr[4]. There is one chiral carbonic atom in the molecular structure of CPG (Figure 1).

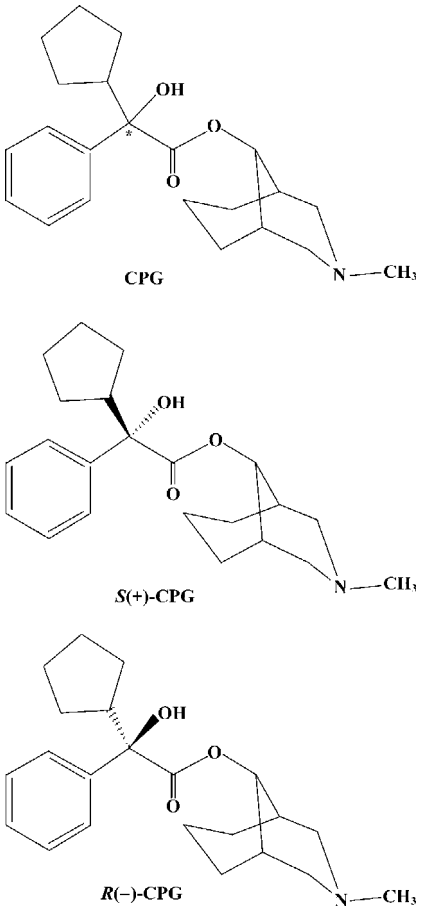

Figure 1. Chemical structure of CPG and its optical isomers.

Thus, there were two optical isomers of CPG with *R*(–)- and *S*(+)-different configurations. The pharmacological effects of these isomers remained unknown. In order to study the pharmacological differences between the stereoisomers and develop more safe drugs, we investigated the pharmacological characteristics of these two chiral muscarinic antagonists by comparing their effects on muscarinic receptors *in vivo* and *in vitro*.

Materials and methods

Chemicals [^{3}H]QNB [^{3}H-quinuclindiny benzilate] (43.3 Ci/mmol) was purchased from Amersham Co (Uppsala, Sweden) (TRK604). CPG and its isomers were synthesized at our institute. CPG comprised nearly the same proportion for *R*(–)- and *S*(+)-CPG. Atropine, pentobarbital sodium, oxotremorine, and carbachol were from Sigma Co (St Lowis, USA).

Animals The experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. Animals used in the study were: male or female Wistar rats weighing 180–220 g, [Grade II, Certificate No scxk (Jing) 2002-0003] and male guinea pigs (200–300 g, Grade II, Certificate No (Jing) 2003-0005), which were purchased from Lianhelihua Co (Beijing, China); Kunming species mice [18–22 g, Grade II, Certificate No SCXK-(Army) 2002-001] were provided from the animal center at our institute.

Binding assays on rat cerebral cortex homogenate Male or female Wistar rats were killed by decapitation. The cerebral cortex was immediately removed and processed as described by Yamamura and Snyder[5]. Protein concentration was determined by the method of Lowry *et al*[6]. Homogenate (50 mg of protein) was incubated for at 37 ºC for 30 min in 0.5 mL of assay buffer containing 6 nmol/L [^{3}H]QNB and various concentrations of drugs. In saturation binding assays, the homogenate was incubated in the presence of [^{3}H]QNB (0.25–20 nmol/L). Non-specific binding was defined as binding in presence of atropine (1 μmol/L). Each sample was filtered through GF/C glass fibers with a vacuum. The filters were rinsed three times with 3 mL cold buffer, and placed in scintillation vials containing 3 mL of scintillation fluid. Radioactivity trapped on the filters was determined by liquid scintillation spectrometry at approximately 40%–50% efficiency.

Carbachol-induced contraction Male guinea pigs were killed by cervical dislocation. The organs required were set up rapidly under 1 g of tension in 20 mL organ baths containing physiological salt solution (PSS), which was kept at 37 ºC and aerated with 5% CO_2 and 95% O_2. Two-centimeter-long portions of terminal ileum were taken at about 5 cm from the ileum-cecum junction and mounted in PSS at 37 ºC. The composition of PSS was as the following (mmol/L): NaCl (118), $NaHCO_3$ (23.8), KCl (4.7), $MgSO_4 \cdot 7H_2O$ (1.18), KH_2PO_4 (1.18), $CaCl_2$ (2.52), and glucose (11.7). Tension changes were recorded isotonically. Tissues were equilibrated for 90 min before the experiments began.

The concentration was increased in a stepwise manner after the response to the previous concentration had reached a plateau to make concentration-response curve for carbachol. After cumulative concentration-response curves were generated in the absence of any antagonist, the ileum strips were washed several times with PSS and allowed to relax to baseline. After 60 min, the strips were incubated with *R*(–)-, *S*(+)-, or CPG for 10–15 min. The concentration-response curves for carbachol were then obtained in the presence of increasing concentrations of different antagonists.

To assess the potency of the antimuscarinic action, the ratio of the ED_{50} values for the carbachol-induced contrac-

tions in the presence and in the absence of antagonist were obtained. Schild plots were obtained by plotting logarithmic (dose ratio-1) against the logarithmic molar concentration of the antagonist, and pA_2 values were derived from the Schild plots according to the method described by Arunlakshana and Schild[7].

Effect on sub-threshold hypnotic dose of sodium pentobarbital induced-sleeping Four dosage groups were used for each drug and each group consists of 10 mice of each sex. CPG and its optical isomers were injected intra-peritoneally (ip). Fifteen minutes later, subthreshold hypnotic dose of sodium pentobarbital (30 mg/kg) was applied ip and lossing in the righting reflex was observed as the score to present the central inhibitory effect of the drugs. The ED_{50} values of these three drugs were estimated to compare the central inhibitory effect of the indicated agents.

Inhibiting oxotremorine-induced salivation Four dosage groups were used for each compound and each group consisted of 10 mice of each sex. CPG and its optical isomers were applied ip 15 min prior to the use of oxotremorine (3 mg/kg) subcutaneously (sc). ED_{50} values were utilized to evaluate the anti-secretive potencies of above described compounds.

Statistics

Binding assay The IC_{50} values were obtained from at least three separate experiments performed in triplicate with between 6 and 8 different concentrations of drugs. Hill coefficients and IC_{50} values were determined using the ORIGIN6.0 software program and inhibition constants (K_i values) were calculated utilizing the Cheng-Prusoff equation[8].

Functional assay In carbachol induced-contraction, the E_{max} value (the maximum contractile response) was obtained from the maximum stress developed, and the ED_{50} value was calculated from a semi-logarithmic plot of the percentage of the maximum response versus drug concentration. Statistical analyses for comparison between groups and between concentration-response curves were performed using analysis of variance (ANOVA). $P<0.05$ was considered statistically significant. In the experiments for observing the effects of CPG and its optical isomers on salivation and sedation induced by oxotremorine and sodium pentobarbital, ED_{50} values were calculated utilizing the Bliss method. Data were shown as mean±SD.

Results

Competitive binding of CPG and its optical isomers to rat central muscarinic acetylcholine receptors The K_d values for [^{3}H]QNB binding to receptors were 6.66±0.95 nmol/L. The B_{max} values were 0.758± 0.086 fmol/mg. The competition binding potency of *R*(–)-CPG for [^{3}H]QNB corresponded to a K_i value of 46.49±1.27 nmol/L (n=4). An average Hill coefficient (n_H) was 1.54±0.06. The affinity of *R*(–)-CPG at central muscarinic acetylcholine receptors was greater than that of CPG (K_i=271.37±72.3 nmol/L, n_H=1.48). *S*(+)-CPG displayed the lowest affinity to muscarinic receptor (K_i=1263.12±131.64 nmol/L, n_H=1.12). The results showed that the isomer with *R*(–)-configuration was more potent than the isomer with *S*(+)-configuration and CPG (Figure 2).

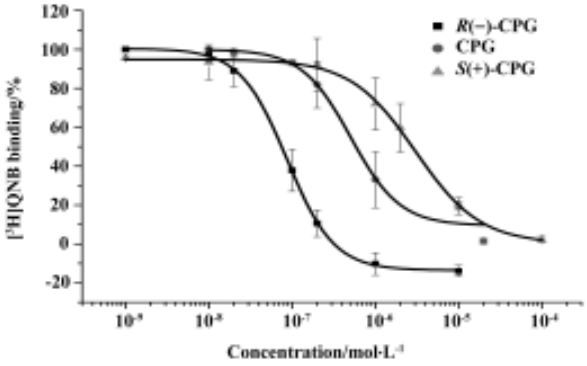

Figure 2. Effects of CPG and its optical isomers on the binding of [^{3}H]QNB to rat central muscarinic acetylcholine receptors. Rat cerebral cortex homogenate was incubated with 6 nmol/L [^{3}H]QNB at 37 °C for 30 min in the absence and presence of increasing concentrations of different drugs. Data were the means from four independent experiments performed in duplicate.

Effect of CPG and its optical isomers on carbachol-induced contraction Carbachol (1×10^{-8}–1×10^{-2} mol/L) caused concentration-dependent contraction of guinea pig ileum. The E_{max} values for the carbachol-induced contractions were 2.9±0.2 g (n=30). CPG and *R*(–)-configuration (1×10^{-8}–1×10^{-7} mol/L) caused typical rightward shifts in the concentration-response curves for carbachol, except for a higher concentration (1×10^{-6} mol/L) of *R*(–)- and CPG, which caused decreases of about 80% of the maximum contractile responses to carbachol. However, *S*(+)-CPG only caused decreases of about 10%–20% of the maximum contractile effects induced by carbachol at the dose of 1×10^{-6} mol/L, the difference was not significant ($P>0.05$). All slopes of the regression lines of Schild plots were close to unity in Figure 3. The IC_{50} value of *R*(–)- and CPG are shown in Figure 4. The rank order of pA_2 values was: CPG (6.80)≈*R*(–)-CPG (6.84) (Table 1).

Potentiating the effect of sub-threshold hypnotic dose of sodium pentobarbital Pentobarbital (ip 30 mg/kg) alone did not cause loss in righting reflex in mice (n=50). Pretreatment

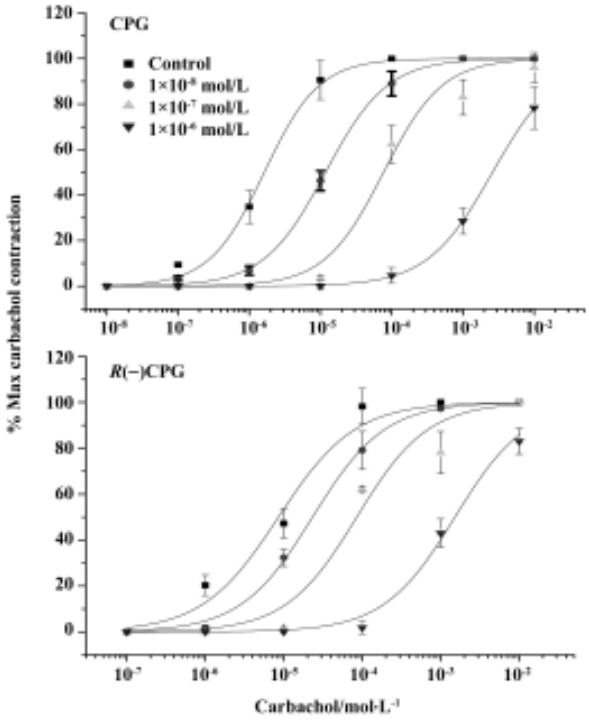

Figure 3. Effects of CPG and *R*(–)-CPG 1 (1×10^{-8}, 1×10^{-7}, and 1×10^{-6} mol/L) on the concentration-response curves for carbachol in guinea ileum. For each experiment, contractile responses were expressed as percentages of the maximum contractile response in the absence of any antagonist. Each point represented the mean±SD of the results from six separate experiments.

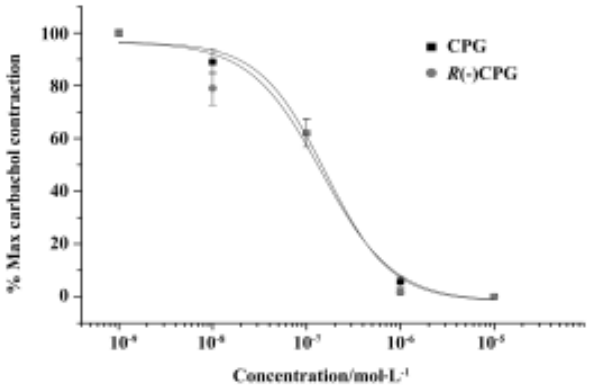

Figure 4. Concentration-response curves for *R*(–)-CPG and CPG (1×10^{-9}–1×10^{-5} mol/L) on the maximum contractile response induced by carbachol (1×10^{-4} mol/L) in guinea pig ileum. For each experiment, contractile responses were expressed as percentages of the maximum contractile response in the absence of any antagonist. Each point represented the mean±SD of the results from six separate experiments; if not shown, SD bars fall within the size of the symbol.

with CPG (14.28–41.64 mg/kg) at 15 min intervals potentiated

Table 1. pA_2 Values and slopes of CPG and *R*(–)-CPG inhibiting carbachol-induced contraction in guinea pig ileum. *n*=6. Mean±SD.

Chiral compound	pA_2	Slopes
CPG	6.80±0.22	1.00±0.05
R(–)-CPG	6.84±0.24	0.95±0.01

the effect of sub-threshold hypnotic dose of sodium pentobarbital in a dose-dependent manner (Table 2). The ED_{50} value and its 95% confident limits of CPG was 21.06 (18.02–24.10) mg/kg. The isomer with *R*(–)- and *S*(+)-configuration did not show any effects on pentobarbital induced-sleeping at the dose from 10.00–29.15 mg/kg. The result suggested that the central depressant effect of CPG was more potent than the other two isomers used separately.

Table 2. The effect of CPG and its optical isomers on sub-threshold hypnotic dose of sodium pentobarbital induced-sleeping. For each experiment, the rate of loss in righting reflex was expressed in the ratio of mice lost in righting reflex to 10 mice.

Chiral compound	Dose /mg·kg^{-1}	The rate of loss in right reflex /%	ED_{50}±95% LC /mg·kg^{-1}
CPG	14.28	1	21.06±3.04
	20.40	0.8	
	29.15	0.5	
	41.64	0.1	
R(–)CPG	10.00	0	
	14.28	0	
	20.40	0	–
	29.15	0	
S(+)CPG	10.00	0	
	14.28	0	
	20.40	0	–
	29.15	0	

Oxotremorine (sc 3mg/kg) induced an obvious salivation in mice (*n*=50). Whereas, CPG and its optical isomers showed antagonistic effects on oxotremorine-induced salivation in dose-dependent manner when pre-administered. The ED_{50}±95% LC for CPG, *R*(–)-, and *S*(+)-configuration were 1.07±0.15, 1.10±0.28, and 16.69±4.82 mg/kg, respectively, which indicated that CPG was equivalent to *R*(–)-CPG and more potent than *S*(+)-CPG in inhibiting glandular secretion (Table 3).

Table 3. Effect of CPG and its optical isomers on oxotremorine-induced salivation. (For each experiment, the rate of anti-salivation was expressed as the ratio of mice lost in righting reflex to 10 mice).

Chiral compound	Dose /mg·kg^{-1}	The rate of anti-salivation	ED_{50}±95% LC /mg·kg^{-1}
CPG	1.68	0.90	1.07±0.15
	1.18	0.70	
	0.82	0.30	
	0.58	0.00	
R(–)-CPG	1.68	0.70	1.10±0.28
	1.18	0.60	
	0.82	0.40	
	0.58	0.10	
S(+)-CPG	20.4	0.80	16.69±4.82
	14.28	0.50	
	10.00	0.30	
	7.00	0.00	

Discussion

Motion sickness is a common disease in modern society. The pathogenic mechanism inducing the sickness is not fully understood. However, the etiologic theory that cholinergic hyperfunction of the vestibular system excites the vomiting center and the central cholinergic neuron system plays an important role in the neural mechanism of motion sickness is generally accepted[9,10]. The anticholinergic agents, such as scopolamine, when used for preventing motion sickness, present some disadvantages at the effective dose, especially the troublesome central inhibitory effect[11].

The new central anticholinergic drug CPG has been widely used in clinic. Animal experiments and clinic research have demonstrated that CPG was more potent and had lesser central inhibitory side effects in the prevention of motion sickness (airsickness and seasickness) than those of central cholinergic drugs, such as scopolamine HCl and dimenhydrinate[12]. There was the same proportion of two enantiomers in the race mixture of CPG. In order to illuminate the pharmacological profiles of its optical isomers, we compared the affinity of CPG and its optical isomers to muscarinic acetylcholine receptors. In the competitive binding assay, it was found that *R*(–)-CPG inhibited the binding of [^{3}H]QNB with the highest potency (K_i=46.49±1.27 nmol/L) compared with CPG (K_i=271.37±72.3 nmol/L) and *S*(+)-CPG (K_i= 1263.12±131.64 nmol/L). In the functional study, CPG and *R*(–)-CPG (1×10^{-8}–1×10^{-6} mol/L) caused parallel rightward shifts of the concentration-response curves for carbachol-induced ileum contraction. All the slopes of the regression lines of Schild plots were close to unity, which implied a competitive antagonism. *S*(+)-configuration slightly decreased the maximum contractile response at the dose of 1×10^{-6} mol/L. The order of potencies of these agents to inhibit the contractile responses was *R*(–)-CPG≈CPG>*S*(+)-CPG. The same result was obtained in inhibiting glandular secretion. These results revealed that *R*(–)-CPG acted as an active composition of racemate with competitive antagonistic mechanism to muscarinic acetylcholine receptors, but *S*(+)-CPG less bioactivity. It also had been to be noted that there was 50% of *S*(+)-CPG with lower binding affinity in racemate CPG; according to binding assay, CPG should less potent in suppressing smooth muscle contraction and glandular secretion. These results suggest that *S*(+)-configuration may increase the potencies of its enantiomer in some manner. Furthermore, at the same dose, *S*(+)- and *R*(–)-configuration did not display any synergestic effect on sub-threshold hypnotic dose of sodium pentobarbital, but their racemate, CPG, revealed remarkable central sedation effects. One possible explanation for these results was that *S*(+)-configuration might play a role in modulating the binding of *R*(–)-configuration by allosteric mechanism. In contrast, muscarinic acetylcholine receptors (mAChRs) modulate the activity of an extraordinarily large number of physiological functions. Individual members of the mAChR family (M_1–M_5) are expressed in a complex, overlapping fashion in most tissues and cell types. The M_1 and M_3 subtypes are the major muscarinic acetylcholine receptors in the salivary gland and M_3 is reported to be more abundant[13,14]. Guinea pig ileum smooth muscle is enriched with muscarinic receptors, the majority of which are of the M_2 subtype whereas the remaining minority belongs to the M_3 subtype[15,16]. The M_1, M_2, and M_4 subtypes of mAChRs are the predominant receptors in the CNS[17]. Our experiments were performed in different species and tissue *in vivo* and *in vitro*, preferential binding of one isomer to muscarinic subtype receptor may cause differences in pharmacological action. Drug enantiomers have identical properties in an achiral environment, but should be considered as different chemical compounds. This is because they often differ considerably in potency, pharmacological activity, and pharmacokinetic profile, since the modules with which they interact in biological systems are also optically active. Interactions of both isomers may differ at the active sites through which pharmacological action is mediated. For this, there were possible subtype and sterochemical selective mechanisms that account for the different actions and levels of activity of the CPG and its enantiomers. Hence, further studies were necessary to resolve the underlying mechanisms of muscarinic receptor with these compounds.

Taken together, the present work demonstrated that *R*(–)-CPG acted as an active component in racemate and a competitive antagonist to acetylcholine muscarinic receptors, but *S*(+)-CPG displayed less activities in comparison to *R*(–)-CPG and its racemate. In contrast to its racemate, both of the enantiomers showed lower central depressant effects.

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