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Characterization of a new muscarinic receptor antagonist PNU-171990 in guinea pig, cat and human smooth muscle

Ali-Reza Modiri [a,*], Mervi Vasänge [a], Peteris Alberts [a], Sukhwinder S. Jossan [b], Staffan Sundquist [c], Per-Göran Gillberg [b]

[a] *Department of Biology, Biovitrum, UF5-1, SE-751 37, Uppsala, Sweden*
[b] *Department of Pharmacology, Pharmacia, Uppsala, Sweden*
[c] *Apoteksbolaget, Jönköping, Sweden*

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Abstract

The present study was done to characterize a new compound, PNU-171990, 2-diisopropyl aminoethyl 1-phenylcyclopentane carboxylate hydrochloride, with functional smooth muscle selectivity at least as high as tolterodine. In vitro homogenates of guinea pig cerebral cortex, parotid gland, heart, urinary bladder, and Chinese hamster ovary (CHO) cells expressing human muscarinic m_1–m_5 receptors PNU-171990 did not show selectivity for any subtype (pK_i, 7.72–8.64). PNU-171990 caused a parallel shift in the concentration–response curve for carbachol-induced contraction of smooth muscle from guinea pig bladder (pK_B, 7.65), guinea pig ileum (pK_B, 8.48), and human ileum (pK_B, 7.10). In vivo PNU-171990 inhibited urinary bladder contraction with a significantly lower ID_{50} than on the salivary secretion (206 and 706 nmol/kg, respectively, $P<0.05$). In conclusion, PNU-171990 is a competitive and potent muscarinic receptor antagonist in vitro with a numerically better selectivity ratio for the bladder contraction over salivation in vivo than tolterodine.

Keywords: Bladder, overactive; Irritable bowel syndrome; Urinary bladder; Ileum

1. Introduction

Agents with affinity to muscarinic receptor subtypes M_2 and M_3 have therapeutic potential for treatment of disorders associated with altered smooth muscle contractility or tone, such as overactive bladder and irritable bowel syndrome.

At present, muscarinic receptor antagonists causing relaxation of smooth muscle are the most widely used treatment for overactive bladder (Wein, 1995) and irritable bowel syndrome (De Point and Malagelada, 1998). However, with the exception of tolterodine, currently used drugs lack functional selectivity for smooth muscle and side effects may limit their usefulness.

The pharmacologically defined muscarinic receptors (M_1–M_5), encoded by five genes (m_1–m_5), are widely distributed in the body (Caulfield and Birdsall, 1998; Hegde and Eglen, 1999). In smooth muscle, activation of the M_3 subtype appears to mediate direct contraction, and activation of the M_2 subtype elicits an indirect contraction by counteracting cyclic AMP-mediated relaxation (Hegde et al., 1997). Since tolterodine, a non-selective muscarinic receptor antagonist, has been shown to have better efficacy than subtype selective muscarinic receptor antagonists (Nilvebrant, 2000), combined blockade of muscarinic M_2 and M_3 receptors seems to be optimal.

The present study was done to characterize a new compound, PNU-171990, 2-diisopropyl aminoethyl 1-phenylcyclopentane carboxylate hydrochloride (Fig. 1), with functional smooth muscle selectivity at least as high as tolterodine for treatment of diseases where a muscarinic receptor antagonist effect is beneficial.

2. Materials and methods

2.1. Radioligand binding studies

Competition binding at muscarinic receptors was determined with (*l*)-(−)-quinuclidinyl(phenyl-4,4′-[^{3}H])benzilate

* Corresponding author. Tel.: +46-8-697-3836; fax: +46-8-697-3832.
E-mail address: ali-reza.modiri@biovitrum.com (A.-R. Modiri).

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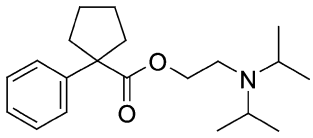

Fig. 1. Chemical structure of the new muscarinic receptor antagonist PNU-171990, an achiral ester, 2-diisopropyl aminoethyl 1-phenylcyclopentane carboxylate hydrochloride.

(1 nM, [^{3}H]QNB, Amersham Pharmacia Biotech) in male guinea pig (Dunkin Hartley, 300–500 g, Charles River, and National Veterinary Institute, Uppsala) tissue homogenates and in Chinese hamster ovary (CHO) cells expressing human muscarinic receptor subtypes (m_1–m_5) (Nilvebrant et al., 1997). Incubation (25 °C) under equilibrium conditions was with urinary bladder (60 min), parotid gland (210 min), heart (100 min), cortex (80 min), and CHO cell homogenates (330 min; 37 °C).

Non-specific binding was defined in the presence of atropine (10 μM). Incubations were done in 96-well microtiter plates and rapidly filtered on GF/B plates. Radioactivity was measured in a Packard TopCount™ scintillation spectrometer.

The antagonist concentration that inhibited 1 nM [^{3}H]QNB binding by 50% (IC_{50}) was calculated by non-linear regression analysis ("Add-in" to Microsoft Excel, XL-fit, ID Business Solutions, Surrey, UK). The ligand concentration, L, of [^{3}H]QNB was measured for each experiment and no significant concentration change was detected. The apparent dissociation constant, K_d, was calculated using the Scatchard plot of saturation data. At least 12 concentrations (0.04–2 nM) of [^{3}H]QNB were assayed to define the dissociation constant. The apparent K_d values from these assays were plotted versus receptor concentration, calculated as B_{max} (protein concentration). The intercept of the abscissa of this curve was defined as K_d. The calculated K_d (pM) values for muscarinic receptors m_1–m_5 were 12 ± 2, 24 ± 1, 10 ± 3, 18 ± 1, and 31 ± 2, respectively. K_i values were calculated from IC_{50}, $K_i = IC_{50}/(1+(L/K_d))$, assuming competitive inhibition (Cheng and Prusoff, 1973).

2.2. Functional in vitro studies

Muscarinic receptor antagonist potency was determined in male guinea pig (350–790 g) urinary bladder (Nilvebrant et al., 1997), guinea pig ileum longitudinal muscle (Alberts et al., 1982), and human ileum (female patients, 39.8 ± 4.4 years old; range 28–58 years; $n = 8$; male patients, 41.0 ± 3.5 years old; range: 32–47 years; $n = 4$). The patients with morbid obesity underwent gastric by pass surgery had not taken drugs that interact with muscarinic receptors (approved by the Ethical Committee of Uppsala University, 15 October 1997, No. 97364). Pieces of ileum up to 3 cm wide and 5 cm long were tied at both ends and put in physiological Krebs–Henseleit solution, containing (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 20, NaH_2PO_4 1.2, D-glucose 11, and hexamethonium 0.1 (Nordström et al., 1983) at room temperature. The human ileum was cut open longitudinally and the mucosa was removed.

Preparations were mounted in organ baths under a passive resting tension of 5 mN (human ileum, guinea pig bladder) and 10 mN (guinea pig ileum). The solution was aerated with 5% CO_2 in O_2 to give pH 7.4 at 37 °C. Agonist concentration–response curves using the muscarinic agonist carbachol were constructed. Antagonist was incubated for 15 min (ileum) and 60 min (bladder), and a second agonist concentration–response curve was constructed.

EC_{50} values calculated from each concentration–response curve using non-linear analysis (Fig.P, Biosoft, UK) were used to calculate pK_B values (Alberts et al., 1999; Kenakin, 1997).

2.3. In vivo study

The muscarinic receptor antagonist effect was studied in female European shorthaired cats (6–12 months old, weight 2.7 ± 0.7 kg) (Nilvebrant et al., 1997). Initial anesthesia was with pentobarbital (35–46 mg/kg i.p.). α-Chloralose (about 1 mg/kg) (190–318 μl/kg/h) was constantly infused in a saphenous vein throughout the experiment.

An incision was made along the mid-line, linea alba, of the abdomen. In order to denervate the urinary bladder, the hypogastric nerves in the intestinal mesenterium and both right and left pelvic nerves dorsal to the urinary bladder were identified and cut. The mid-urethra was opened and a catheter PE 240 (Clay Adams, NJ, USA) was inserted through the mid-urethra into the urinary bladder. This catheter was connected to a Grass P23 ID pressure transducer and Grass Model 7D polygraph (MA, USA). The right femoral artery was catheterized with a polyethylene tube (PE 50), which was inserted about 7 cm from the femoral triangle towards the heart and used for administration of acetylcholine. Bladder contraction measured isovolumetrically was evoked by intraarterial injection of submaximal acetylcholine dose (1–4 μg/kg) 1 min before and 9 min after administration of each test compound dose.

The left or right parasympathetic lingual nerve was exposed below the submandibular ganglion and cut as far

Table 1
Inhibition constants (pK_i) calculated for guinea pig tissues

Compound	Bladder (M_2, M_3)	Cerebral cortex (M_1, M_2, M_4)	Heart (M_2)	Parotid gland (M_3)
PNU-171990	7.37 ± 0.08	8.64 ± 0.02[a,b]	7.84 ± 0.07	7.90 ± 0.11
Tolterodine[c]	8.53 ± 0.03	9.12 ± 0.01	8.80 ± 0.01	8.32 ± 0.03
Atropine	8.57 ± 0.1	9.51 ± 0.03[b]	8.82 ± 0.01	8.90 ± 0.12

Data are expressed as mean ± S.E.M. of three to four determinations.
[a] Significantly different from parotid gland and cerebral cortex ($P < 0.05$).
[b] Significantly different from heart ($P < 0.05$).
[c] Data from Nilvebrant et al. (1997).

Table 2
Dissociation constants (pK_i) and Hill coefficients (n_H) calculated for human muscarinic receptors m_1–m_5 expressed in CHO cells

		m_1	m_2	m_3	m_4	m_5
PNU-171990	pK_i	7.72 ± 0.16	7.19 ± 0.23	7.60 ± 0.21	7.61 ± 0.29	7.69 ± 0.16
	n_H	0.93 ± 0.40	1.02 ± 0.50	0.81 ± 0.12	0.74 ± 0.28	0.97 ± 0.32
Tolterodine[a]	pK_i	8.52 ± 0.03	8.42 ± 0.08	8.47 ± 0.10	8.30 ± 0.07	8.47 ± 0.10
	n_H	1.03 ± 0.04	1.00 ± 0.04	1.06 ± 0.03	1.05 ± 0.07	1.00 ± 0.05
Atropine	pK_i	8.73 ± 0.15	8.06 ± 0.19	8.99 ± 0.31	8.71 ± 0.33	8.53 ± 0.27
	n_H	0.80 ± 0.32	0.98 ± 0.16	1.31 ± 0.29	1.17 ± 0.14	1.21 ± 0.40

Data are expressed as mean ± S.E.M. of three to seven experiments.
[a] Data from Nilvebrant et al. (1997).

proximally and dorsally as possible. The salivary ducts were exposed close to the submandibular ganglion. The median duct, that of the submandibular gland, was cannulated with a catheter (PE 10) and the retrolingual duct was ligated. The peripheral nerve stump of the lingual nerve was placed on a bipolar platinum ring electrode and moistened with physiological saline. Salivation was induced by submaximal lingual nerve stimulation (6 V, 2 ms, 5 Hz, 2.0 min periods) with a bipolar platinum ring electrode connected to a Grass S48 Stimulator (MA, USA), 7 min before and 7 min after administration of each dose.

PNU-171990 (0.03–3 mg/kg) or saline was given at 1 ml/kg/min. ID_{50} values were determined from each individual dose–response curve by linear regression (Nilvebrant et al., 1997). Statistical analysis was performed using Student's *t*-test.

The recommendations from the Declaration of Helsinki have been adhered to. All experiments were approved by the ethical committee.

3. Results

3.1. Radioligand binding studies

PNU-171990 and atropine inhibited the specific binding of [^{3}H]QNB in homogenates of guinea pig urinary bladder, cerebral cortex, heart and parotid gland (Table 1), and in

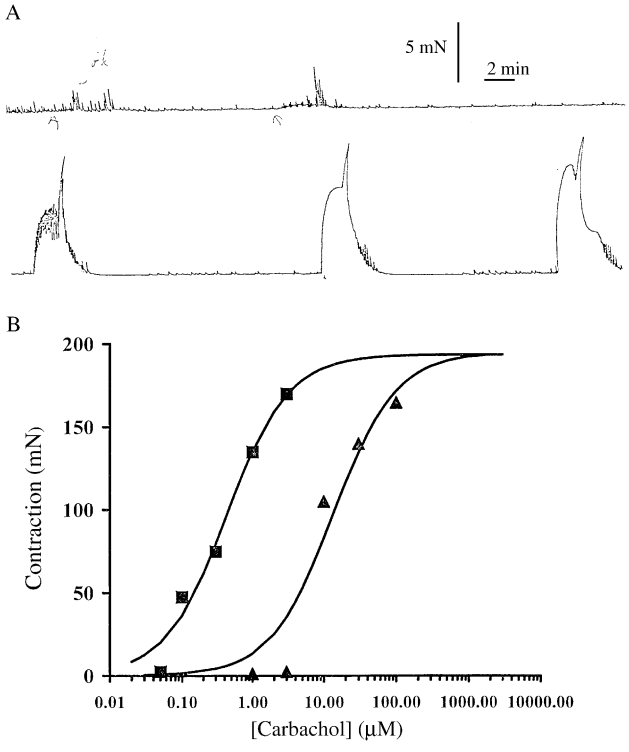

Fig. 2. Contractile responses of the isolated human ileum longitudinal muscle to increasing doses of carbachol are shown (A). Plot of two concentration–response curves obtained in an isolated preparation of the human ileum longitudinal muscle in the absence (■) and presence (▲) of PNU-171990 (1 μM) (B).

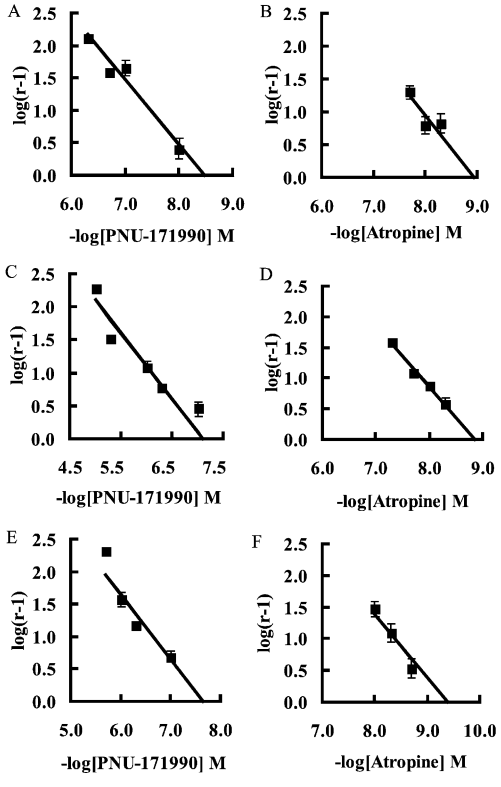

Fig. 3. Schild plots for PNU-171990 and atropine in the isolated guinea pig ileum (A, B), human ileum (C, D), and guinea pig urinary bladder (E, F). For *n* values, see Table 3.

Table 3
Cholinergic muscarinic receptor antagonist pK_B values determined from contraction measurements in the isolated muscle strips

Compound	Guinea pig urinary bladder		Guinea pig ileum		Human ileum	
	pK_B	n	pK_B	n	pK_B	n
PNU-171990	7.65 ± 0.062	10	8.48 ± 0.071	11	7.10 ± 0.063	13
Atropine	9.39 ± 0.076	9	8.95 ± 0.081	10	8.86 ± 0.029	10

Data are expressed as mean ± S.E.M.

cloned human receptors (Table 2) in a concentration-dependent manner.

3.2. Functional in vitro studies

Concentration–response curves using carbachol were constructed (Fig. 2). The calculated EC_{50} values for carbachol were in the guinea pig ileum (0.31 ± 0.043 μM, n = 46, N = 16) and human ileum (0.95 ± 0.084 μM, n = 51, N = 12) longitudinal muscle, where n is number of preparations and the N is number of guinea pigs and patients.

In the guinea pig and human ileum, and the guinea pig urinary bladder, PNU-171990 and atropine caused a parallel shift in the carbachol concentration–response curve, and the Schild plots were linear in the concentration range tested with slopes not significantly different from negative unity (Fig. 3; Table 3).

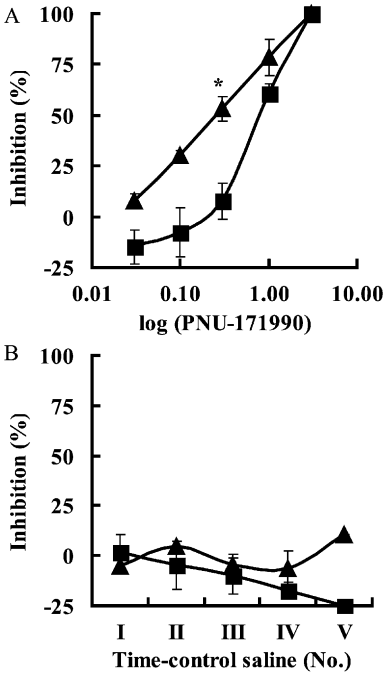

Fig. 4. Dose–response curves in the cat in vivo on the intraarterial ACh-induced urinary bladder contraction (▲, 1–4 μg/kg), and salivary secretion (■) induced by submaximal lingual nerve stimulation (6 V, 2 ms, 5 Hz, 2.0-min periods) for PNU-171990 (A), and time-control saline (B). Results are expressed as percentage inhibition of the maximal response in each experiment and are the mean ± S.E.M. (n = 4). Statistical difference (P) between bladder contraction and salivation is * P < 0.05.

Table 4
ID_{50} values (mean ± S.E.M., n = 4) for the effect of PNU-171990 on urinary bladder contraction and salivary secretion in the cat in vivo

Compounds	Urinary bladder contraction	Salivary secretion	Selectivity ratio
	ID_{50} (nmol/kg)	ID_{50} (nmol/kg)	(Salivation/contraction)
PNU-171990	706 ± 113	2062 ± 367	2.9[b]
Tolterodine[a]	101 ± 8	257 ± 44	2.5[b]
Atropine[a]	18 ± 4	21 ± 4	1.2[NS]

Statistical difference (P) between ID_{50} values is as follows: [NS]P > 0.05, [b]P < 0.05.
[a] Data from Nilvebrant et al. (1997).

3.3. In vivo study

In the anaesthetized cat, PNU-171990 inhibited both the acetylcholine-induced bladder contraction and the electrically induced salivary secretion in a dose dependent manner (Fig. 4A; Table 4). The effect on urinary bladder contractions occurred at significantly lower doses than effect on saliva secretion (P < 0.05). Administration of saline did not alter bladder contraction or salivation (Fig. 4B).

4. Discussion

The aim of this study was to characterize a new compound with functional smooth muscle selectivity at least as high as tolterodine for treatment of diseases where a muscarinic receptor antagonist effect is beneficial.

The present results show that the new muscarinic receptor antagonist PNU-171990 showed no subtype selectivity for muscarinic receptors from isolated guinea pig tissues or cloned human receptors (Tables 1 and 2). In functional in vitro studies, PNU-171990 antagonized carbachol-induced contractions in smooth muscle from guinea pig urinary bladder, and guinea pig and human ileum (Table 3; Fig. 3), yielding pK_B values similar to the pK_i values. PNU-171990 caused a parallel shift in the concentration–response curve for carbachol Schild plot slopes were not significantly different from unity, suggesting that PNU-171990 bound to a single receptor site in a competitive manner.

The present in vivo cat model has previously been shown to be predictive for tissue selectivity of a muscarinic receptor antagonist, tolterodine, in man (Nilvebrant, 2001; Nilvebrant et al., 1997). PNU-171990, like tolterodine, has no selectivity for any muscarinic receptor subtype. In addition, both compounds show functional tissue selectivity for urinary bladder contraction over salivary secretion in the anaesthetized cat in vivo. Thus, PNU-171990 showed significant tissue selectivity for bladder contraction over salivary secretion, yielding a numerically higher selectivity ratio than tolterodine (Table 4).

In a recent clinical paper (Van Kerrebroeck et al., 2001), an improved bladder selectivity for tolterodine was demonstrated with an extended release formulation. This is probably due to lower serum peak concentrations giving even less incidence of dry mouth. Accordingly, pharmacokinetic parameters may also contribute to the in vivo PNU-171990 tissue selectivity.

In conclusion, PNU-171990 is a competitive and potent muscarinic receptor antagonist in vitro with a numerically better selectivity ratio for the bladder contraction over salivation in vivo than tolterodine.

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References

Alberts, P., Bartfai, T., Stjärne, L., 1982. The effects of atropine on ^{3}H-acetylcholine secretion from guinea-pig myenteric plexus evoked electrically or by high potassium. J. Physiol. 329, 93–112.

Alberts, P., Bergström, P.A., Fredrickson, M.G., 1999. Characterisation of the functional alpha-adrenoceptor subtype in the isolated female pig urethra. Eur. J. Pharmacol. 371, 31–38.

Caulfield, M.P., Birdsall, N.J., 1998. International Union of Pharmacology: XVII. Classification of muscarinic acetylcholine receptors. Pharmacol. Rev. 50, 279–290.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

De Point, F., Malagelada, J.R., 1998. Functional gut disorders: from motility to sensitivity disorders. A review of current and investigational drug for their management. Pharmacol. Ther. 80, 49–88.

Hegde, S.S., Eglen, R.M., 1999. Muscarinic receptor subtypes modulating smooth muscle contractility in the urinary bladder. Life Sci. 64, 419–428.

Hegde, S.S., Choppin, A., Bonhaus, D., Briaud, S., Loeb, M., Moy, T.M., Loury, D., Eglen, R.M., 1997. Functional role of M_2 and M_3 muscarinic receptors in the urinary bladder of rats in vitro and in vivo. Br. J. Pharmacol. 120, 1409–1418.

Kenakin, T., 1997. Pharmacologic Analysis of Drug–Receptor Interaction, 3rd ed. Lippincott-Raven, Philadelphia, pp. 232–339.

Nilvebrant, L., 2000. The mechanism of action of tolterodine. Rev. Contemp. Pharmacother. 11, 13–27.

Nilvebrant, L., 2001. Clinical experiences with tolterodine. Life Sci. 68, 2549–2556.

Nilvebrant, L., Andersson, K.E., Gillberg, P.G., Stahl, M., Sparf, B., 1997. Tolterodine—a new bladder-selective antimuscarinic agent. Eur. J. Pharmacol. 327, 195–207.

Nordström, Ö., Alberts, P., Westlind, A., Undén, A., Bartfai, T., 1983. Presynaptic antagonist-postsynaptic agonist at muscarinic cholinergic synapses. *N*-methyl-*N*-(1-methyl-4-pyrrolidino-2-butynyl)acetamide. Mol. Pharmacol. 24, 1–5.

Van Kerrebroeck, P., Kreder, K., Jonas, U., Zinner, N., Wein, A., Tolterodine Study, 2001. Tolterodine once-daily: superior efficacy and tolerability in the treatment of the overactive bladder. Urology 57, 414–421.

Wein, A.J., 1995. Pharmacology of incontinence. Urol. Clin. North Am. 22, 557–577.