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Pharmacology & Toxicology
ISSN 0901-9928

Antimuscarinic Potency and Bladder Selectivity of PNU–200577, a Major Metabolite of Tolterodine

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(Received December 4, 1996; Accepted April 24, 1997)

Abstract: PNU–200577 (labcode DD 01 [(R)-N,N-diisopropyl-3-(2-hydroxy-5-hydroxymethylphenyl)-3-phenylpropanamine) is a major pharmacologically active metabolite of tolterodine, a new muscarinic receptor antagonist intended for the treatment of an overactive bladder. *In vitro*, PNU-200577 produced a competitive and concentration-dependent inhibition of carbachol-induced contraction of guinea-pig isolated urinary bladder strips (K_B=0.84 nM; pA_2=9.14). *In vivo*, PNU-200577 was significantly more potent at inhibiting acetylcholine-induced urinary bladder contraction than electrically induced salivation in the anaesthetised cat (ID_{50} 15 and 40 nmol · kg^{-1}, respectively; $P<0.01$). In radioligand binding studies carried out in homogenates of guinea-pig tissues and Chinese hamster ovary cell lines expressing human muscarinic m1–m5 receptors, PNU-200577 was not selective for any muscarinic receptor subtype. Thus, PNU-200577 is similar to tolterodine in terms of antimuscarinic potency, functional selectivity for the urinary bladder *in vivo* and absence of selectivity for muscarinic receptor subtypes *in vitro*. The results of this study clearly indicate that PNU–200577 contributes to the therapeutic action of tolterodine, in view of its high antimuscarinic potency, similar serum concentration and lower degree of protein binding.

Contraction of the human urinary bladder is known to be mediated predominantly through stimulation of muscarinic receptors (Andersson 1993) and, thus, the pharmacological treatment of urinary urge incontinence is based on muscarinic receptor antagonists (Andersson 1988; Wein *et al.* 1994). Tolterodine is a new, potent competitive muscarinic receptor antagonist that has been developed for the treatment of urinary urge incontinence and other symptoms related to an overactive bladder. *In vitro*, tolterodine exhibits a high affinity and specificity for muscarinic receptors (Nilvebrant *et al.* 1994 & 1996). In human isolated urinary bladder preparations tolterodine potently antagonises contractions induced by either carbachol or electrical stimulation (Naerger *et al.* 1995; Nilvebrant *et al.* 1995). *In vivo*, a selectivity of tolterodine for the urinary bladder over salivary glands has been demonstrated in the anaesthetised cat (Gillberg *et al.* 1994; Nilvebrant *et al.* 1996). At least 5 genes encoding for molecularly distinct subtypes of muscarinic receptors (m1–m5) have been cloned and expressed in cell lines and 3 of these subtypes can be distinguished pharmacologically (M_1–M_3) (Caulfield 1993; Hulme *et al.* 1990). However, the selectivity profile of tolterodine *in vivo* cannot be explained in terms of selectivity for one of these muscarinic receptor subtypes (Nilvebrant *et al.* 1996).

Metabolism studies in humans have revealed that tolterodine undergoes oxidation via cytochrome P450 2D6 to a 5-hydroxymethyl derivative, PNU-200577 (labcode DD 01 [(R)-N,N-diisopropyl-3-(2-hydroxy-5-hydroxymethylphenyl)-3-phenylpropanamine) (Andersson *et al.* 1995; Postlind *et al.* 1996). PNU-200577 has been synthesised and found to be pharmacologically active in preliminary experiments. While serum levels of the metabolite in humans are generally comparable to those of tolterodine following oral administration of the parent compound (unpublished observations), tolterodine and PNU-200577 differ in terms of the extent of protein binding. In human and cat serum, PNU-200577 exists as >30% unbound drug while tolterodine is >95% protein bound (unpublished observations). Taken together, these data suggest that PNU–200577 contributes to the therapeutic action of tolterodine. Thus, the aim of this study was to determine the pharmacological profile of PNU-200577 both *in vitro* and *in vivo*. For comparative purposes, previously published data on tolterodine (Nilvebrant *et al.* 1994 & 1996) are included.

Materials and Methods

Functional in vitro studies. Male guinea-pigs (Dunkin Hartley strain) weighing 300–500 g were used in all experiments. Antimuscarinic potency of PNU-200577 was determined in guinea-pig isolated urinary bladder strips, as previously described (Nilvebrant *et al.* 1996). Following equilibration, the urinary bladder preparations were repeatedly exposed to a standard concentration of the muscarinic receptor agonist carbachol (3 μM; EC_{80}). A reproducible control response was established before the effects of PNU-200577 were determined. Following the generation of a cumulative concentration-response curve to carbachol (control), PNU-200577 was added to the tissue bath for 60 min. (Nilvebrant 1986) and a second cumulative concentration-response curve to carbachol generated in the presence of PNU-200577. The effect of PNU-200577 was studied at 4–5 different concentrations using separate tissue preparations. Responses were expressed as a percentage of the maximal

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Radioligand binding studies. The affinity of PNU–200577 for muscarinic receptors was determined by radioligand binding studies in homogenates of guinea-pig tissues (urinary bladder, parotid gland, heart and cerebral cortex) and in Chinese hamster ovary cells expressing the human muscarinic receptor subtypes (m1–m5). Briefly, tissue homogenates were incubated at 25° with the radioligand 1-quinuclidinyl [phenyl-4 ^{3}H] benzilate [(−)^{3}H-QNB] and different concentrations of unlabelled antagonist under conditions of equilibrium: urinary bladder, 60 min. (Nilvebrant & Sparf 1983a); parotid gland, 210 min. (Nilvebrant & Sparf 1982); and heart and cerebral cortex, 80 min. (Nilvebrant & Sparf 1986). Incubations were terminated by centrifugation. The pellets were washed three times with buffer before radioactivity was determined by liquid scintillation spectrometry as previously described (Nilvebrant & Sparf 1983a). Radioligand binding studies in Chinese hamster ovary cells were carried out in culture plates. Briefly, cell homogenates were incubated with (−)^{3}H-QNB and different concentrations of unlabelled antagonist under conditions of equilibrium (37° for 300 min.). Incubations were terminated by rapid filtration and the amount of radioactivity determined by liquid scintillation spectrometry. Total binding of (−)^{3}H-QNB was determined in the absence of any competing ligand, while non-specific binding was determined in the presence of unlabelled atropine (10 μM). Receptor-specific binding in each experiment was defined as total non-specific binding.

In vivo studies. The antimuscarinic effects of PNU-200577 *in vivo* were studied in the anaesthetised cat, as previously described (Nilvebrant *et al.* 1996). Ten female European short-haired cats (2.2–3.1 kg, 8–10 months of age) were used in the experiments (PNU-200577, n=5; control, n=5). Briefly, bladder contractions were elicited by intraarterial administration of a submaximal dose of acetylcholine (1–2 $\mu g \cdot kg^{-1}$), while salivation was induced by supramaximal electrical stimulation (6 V, 2 msec, 5 Hz) of the parasympathetic chorda-lingual nerve over 2 min. Acetylcholine was administered before and approximately 9 and 16 min. after each dose of PNU-200577 (2–203 $nmol \cdot kg^{-1}$ [0.001–0.1 $mg \cdot kg^{-1}$]) or saline, which was administered by intravenous infusion in the right femoral vein at a rate of 1 $ml \cdot min.^{-1} \cdot kg^{-1}$. Electrical stimulation of the chorda-lingual nerve was performed before and approximately 7 min. after each dose of PNU-200577 or saline.

Data analysis. The concentration of carbachol that produced 50% of the maximal contractile response (EC_{50}) in guinea-pig isolated urinary bladder strips was determined in the absence and presence of different concentrations of PNU-200577, respectively. The affinity of PNU-200577 was calculated as the dissociation constant K_B (Schild 1949). In the radioligand binding experiments, the concentration of PNU-200577 that inhibited (−)^{3}H-QNB binding by 50% (IC_{50}) was determined from the experimental concentration-inhibition curves. Dissociation constants (K_i) were calculated by correcting the IC_{50} values for the radioligand-induced parallel shift and the differences in receptor density, using the method described by Jacobs *et al.* (1975); see also Nilvebrant & Sparf (1982 & 1983b) for details. Differences between the inhibitory effect exerted by PNU-200577 on urinary bladder contraction and on salivary secretion in the anaesthetised cat were analysed for each dose using a paired Student's t-test. Differences between the doses of antagonist causing a 50% inhibition of these responses (ID_{50}) were also analysed. All data are expressed as mean±S.E.M. P values <0.05 were considered statistically significant.

Drugs and chemicals. The following drugs and chemicals were used: PNU-200577 [(R)-N,N-diisopropyl-3-(2-hydroxy-5-hydroxymethylphenyl)-3-phenylpropanamine, as the mandelate salt (batch nos. CA 007 075:1 and CA 007 075:3); Pharmacia & Upjohn AB, Sweden]; atropine sulphate, carbachol (carbamylcholine chloride), acetylcholine chloride and phenyl methyl sulphonyl fluoride (Sigma Chemical Company, U.S.A.); (−)^{3}H-QNB (specific radioactivity 38.8–45.4 $Ci \cdot mmol^{-1}$ (1.43–1.63 $Tbq \cdot mmol^{-1}$); Du Pont NEN Research Products, U.S.A.); Dulbecco's modified Eagle's medium and HAM's F12 medium (National Veterinary Institute, Sweden); foetal bovine serum albumin (HyClone Lab, U.S.A.); L-glutamine and penicillin/streptomycin (ICN Biomedicals, U.S.A.). Other chemicals used (analytical grade) were purchased from general commercial sources. (−)^{3}H-QNB was diluted in absolute ethanol. Fresh solutions of PNU-200577, carbachol, atropine and acetylcholine were prepared for each experiment; for functional *in vitro* studies dilutions were made in double distilled water, while for *in vivo* studies solutions were diluted in saline.

Results

Functional in vitro studies.

PNU-200577 produced a concentration-dependent, parallel, rightward shift in the concentration-response curve to carbachol in guinea-pig urinary bladder strips. The maximum response to carbachol was not depressed. The mean K_B value for PNU-200577 was 0.84±0.09 nM (n=20). Schild plot analysis for PNU-200577 determined a pA2 value of 9.14 and a slope that was close to unity (0.99). This indicates that PNU-200577 is a competitive antagonist at the muscarinic receptors in the guinea-pig urinary bladder.

Radioligand binding studies.

PNU-200577 caused a concentration-dependent inhibition of (−)^{3}H-QNB binding in homogenates of guinea-pig urinary bladder, parotid gland, heart and cerebral cortex (table 1). The PNU–200577 concentration-inhibition curves of (−)^{3}H-QNB binding were parallel and had Hill coefficients (n_H) close to unity (table 1), indicating that PNU-200577 binds to a single population of muscarinic binding sites in each tissue.

Table 1.

Dissociation constants (K_i) and Hill coefficients (n_H) for PNU-200577 and tolterodine, as determined by competitive radioligand binding studies in homogenates of guinea-pig tissues.

	PNU-200577			Tolterodine[a]		
Tissue	n	K_i (nM)	n_H	n	K_i (nM)	n_H
Urinary bladder	8	2.9±0.3	0.98±0.07	6	2.7±0.2	1.02±0.03
Parotid gland	7	5.2±0.3	1.06±0.07	5	4.8±0.3	1.04±0.03
Heart	6	1.1±0.1	1.04±0.04	5	1.6±0.04	1.04±0.06
Cerebral cortex	7	0.60±0.04	0.93±0.04	5	0.75±0.01	1.05±0.03

[a] Data from Nilvebrant *et al.* (1996).

Table 2.

Dissociation constants (K_i) and Hill coefficients (n_H) for PNU-200577 and tolterodine at human muscarinic receptors expressed in Chinese hamster ovary cells.

Receptor subtype	PNU-200577		Tolterodine[a]	
	K_i (nM)	n_H	K_i (nM)	n_H
m1	2.3±0.2	1.02±0.03	3.0±0.2	1.03±0.04
m2	2.0±0.5	0.92±0.03	3.8±0.7	1.00±0.04
m3	2.5±0.5	0.93±0.04	3.4±0.8	1.06±0.03
m4	2.8±0.2	0.97±0.04	5.0±0.8	1.05±0.07
m5	2.9±0.4	1.01±0.04	3.4±0.8	1.00±0.05

[a] Data from Nilvebrant *et al.* (1996).
Results are expressed as mean±S.E.M. of 4–6 separate experiments.

PNU-200577 was a potent inhibitor of $(-)^3$H-QNB binding in homogenates of Chinese hamster ovary cells expressing human muscarinic m1–m5 receptors (table 2). Overall, K_i values for PNU-200577 were similar at the respective muscarinic receptor subtypes.

In vivo studies.

Acetylcholine (0.5–8 ($g \cdot kg^{-1}$ intraarterially) produced dose-dependent contraction of the urinary bladder in the anaesthetised cat, with a dose of 1–2 $\mu g \cdot kg^{-1}$ intraarterially producing a reproducible submaximal response (data not shown). PNU-200577 produced dose-dependent inhibition of acetylcholine-induced urinary bladder contraction (fig. 1), with a mean ID_{50} of 15±4 $nmol \cdot kg^{-1}$ (n=5). The threshold dose (ID_{30}) for inhibition of acetylcholine-induced urinary bladder contraction was 7±3 $nmol \cdot kg^{-1}$.

Electrical stimulation of the chorda-lingual nerve induced a mean salivary secretion of 321±31 $\mu l \cdot 2\ min.^{-1}$. PNU-200577 produced dose-dependent inhibition of electrically stimulated salivation (fig. 1) with an ID_{30} and ID_{50} of 21±2 and 40±2 $nmol \cdot kg^{-1}$, respectively ($P<0.05$ and $P<0.01$ versus inhibition of bladder contraction). This indicates that PNU-200577 is three times more potent at the urinary bladder compared to the salivary gland.

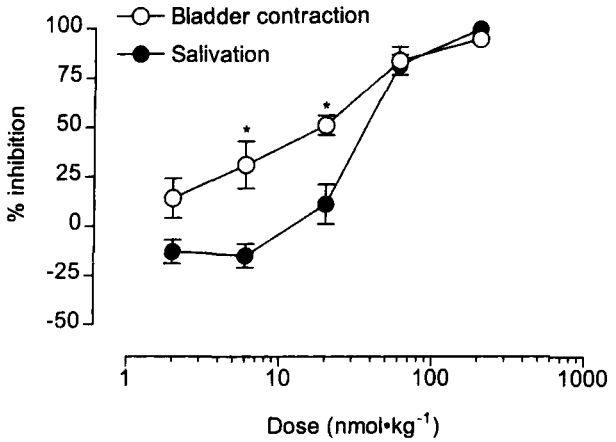

Fig. 1. Effect of PNU-200577 on acetylcholine-induced urinary bladder contraction and electrically stimulated salivary secretion in the anaesthetised cat. Results are expressed as percentage inhibition of the maximum response in each experiment, and are the mean (S.E.M. of 5 separate experiments. *=P<0.05 versus inhibition of

In control animals the administration of saline had only minor effects on the basal response to bladder contraction and salivation. Neither PNU-200577 nor saline had a significant effect on heart rate (data not shown).

Discussion

In the anaesthetised cat, PNU-200577 produced a dose-dependent inhibition of acetylcholine-induced urinary bladder contraction and electrically stimulated salivation, and was almost three times more potent for inhibition of urinary bladder contractions compared with salivation (ID_{50} 15 and 40 $nmol \cdot kg^{-1}$, respectively). It may be argued that it would have been preferable to induce bladder contractions and salivation using an identical means of stimulation, i.e. either electrically or chemically with a muscarinic agonist given intravenously. However, both methods have practical limitations. For example, bladder contractions induced by electrical stimulation of the pelvic nerve are partly resistant to blockade by atropine and other muscarinic receptor antagonists; indeed, the cholinergic component constitutes only about 30% of the response (unpublished observations), while the remainder is non-cholinergic non-adrenergic mediated. This is well known to be the case in animal bladders, as shown in numerous *in vitro* and *in vivo* studies (Andersson 1988 & 1993; Wein *et al.* 1994). However, since it is generally accepted that contractions of the human bladder are mediated mainly by muscarinic receptors (Andersson 1993; Wein *et al.* 1994), we found it more relevant to study only the cholinergic component of the bladder response in the cat. Acetylcholine must be administered close to the target organ in order to avoid degradation. We therefore used intraarterial injections because it is not possible to simultaneously stimulate both the bladder and the salivary glands by intravenous administration of acetylcholine without using doses high enough to kill the animals. We also tried to administer stable analogues of acetylcholine (e.g. carbachol, methacholine) by intravenous injection. In general, however, this method resulted in an unstable baseline bladder response over time, together with low and variable stimulation of salivation. The inhibition exerted by muscarinic antagonists could therefore not be reliably determined and reproduced between animals (data not shown). In the present study, chemical stimulation of the bladder with i.a. acetylcholine and electrical stimulation of the salivary glands were therefore the methods of choice in terms of reliable and consistent responses. Both responses were elicited by the natural transmitter, acetylcholine, but the concentration of acetylcholine at the muscarinic receptors in the bladder and salivary gland is obviously unknown, as always in *in vivo* experiments. Thus, it cannot be excluded that a difference in end-organ activation to some extent can explain the bladder selectivity of PNU-200577. However, in

vivo data on atropine and oxybutynin in previous studies were clearly in line with the general clinical experience with these drugs (Gillberg *et al.* 1994; Nilvebrant *et al.* 1996).

The selectivity profile of PNU-200577 observed in this study is identical to that of tolterodine (Nilvebrant *et al.* 1996), although PNU-200577 is more potent than the parent compound *in vivo*. ID_{50} values for tolterodine were 101 and 257 nmol · kg^{-1}, respectively, for the inhibition of urinary bladder contraction and salivation (Nilvebrant *et al.* 1996). A likely explanation for the higher potency of PNU-200577 *in vivo* is that a very low percentage (<5%) of tolterodine is unbound in serum, whereas >30% of PNU-200577 exists as the unbound drug (unpublished observations). Since the total serum drug concentrations of tolterodine and PNU-200577 are similar following oral administration of the parent compound (unpublished observations), the response observed *in vivo* following oral administration of tolterodine is likely to be, in part, the result of the activity of unbound PNU-200577.

The findings of this study indicate that the pharmacological profile of PNU-200577 *in vitro* is also essentially identical to that of tolterodine (Nilvebrant *et al.* 1996). Indeed, PNU-200577 was a potent and competitive antagonist at muscarinic receptors in guinea-pig isolated urinary bladder strips and exhibited a high affinity for muscarinic receptors in the urinary bladder, parotid gland, heart and cerebral cortex. In addition, PNU-200577 had similar affinity for all five muscarinic receptor subtypes expressed in Chinese hamster ovary cells (K_i 2.0–2.9 nM), as previously shown for tolterodine (K_i 3.0–5.0 nM) (Nilvebrant *et al.* 1996). Thus, as for tolterodine, the selectivity profile of PNU-200577 *in vivo* cannot be attributed to selectivity for a single muscarinic receptor subtype (Nilvebrant *et al.* 1996). It is interestingly to note that oxybutynin, a muscarinic antagonist that exhibits a ten-fold higher selectivity for m3 over m2 receptors *in vitro*, displays the reverse selectivity profile to PNU-200577 and tolterodine in the anaesthetised cat, i.e. oxybutynin inhibits salivation by 80% at doses required for 50% inhibition of the urinary bladder response (Nilvebrant *et al.* 1996). Thus, the reverse selectivity pattern observed for oxybutynin compared with PNU-200577 and tolterodine may be related to the difference in relative affinity for M_3/m3 and M_2/m2 receptors. Other compounds with selectivity for M_3/m3 over M_2/m2 receptors *in vitro*, such as darifenacin and UH-AH 37, exhibit an identical selectivity profile to oxybutynin in the anaesthetised cat (Nilvebrant *et al.* 1996). In contrast, the M2/m2 selective compound AQ-RA 741 was found to exhibit a bladder selectivity *in vivo*, which was similar to that demonstrated for tolterodine (Nilvebrant *et al.* 1996). The data on AQ-RA 741 may suggest a functional role for M_2/m2 receptors in bladder contraction and, thus, in the selectivity for bladder over salivary glands demonstrated for PNU-200577 *in vivo*.

In summary, the pharmacological *in vitro* and *in vivo* profiles of PNU-200577 are almost identical to those of tolterodine, the parent compound. In view of its high antimuscarinic potency, lower degree of protein binding and similar serum concentration in humans after oral administration of the parent compound, PNU-200577 may contribute to the therapeutic action of tolterodine.

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