Demonstration of Muscarinic and Nicotinic Receptor Binding Activities of Distigmine to Treat Detrusor Underactivity

Taketsugu HARADA,[a] Kazumi FUSHIMI,[a] Aya KATO,[a] Yoshihiko ITO,[a] Saori NISHIJIMA,[b] Kimio SUGAYA,[b] and Shizuo YAMADA*,[a]

[a] *Department of Pharmacokinetics and Pharmacodynamics and Global Center of Excellence (COE) Program, School of Pharmaceutical Sciences, University of Shizuoka; 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan: and* [b] *Southern Knights' Laboratory LLP; Okinawa 904–2171, Japan.*

Received December 8, 2009; accepted December 21, 2009; published online January 6, 2010

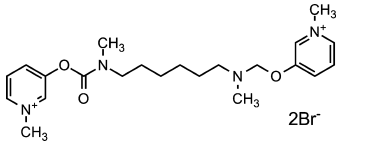
The present study was undertaken to examine whether distigmine, a therapeutic agent used to treat detrusor underactivity, binds directly to muscarinic and nicotinic receptors. We used radioreceptor binding assays and compared the effects of distigmine with those of neostigmine and donepedil. The inhibitory effect of distigmine on the blood acetylcholinesterase (AChE) activity was significantly weaker than that of neostigmine. Distigmine, neostigmine, and donepezil competed for specific binding sites of [*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) and [^{3}H]oxotremorine-M in the bladder, submaxillary gland and cerebral cortex of rats in a concentration-dependent manner, indicating significant binding activity of muscarinic receptors. Distigmine displayed significantly higher affinity for binding sites of [^{3}H]oxotremorine-M compared with those of [^{3}H]NMS as revealed by large ratios of its K_i value for [^{3}H]NMS to that for [^{3}H]oxotremorine-M, suggesting that it has preferential affinity for agonist sites of muscarinic receptors. Distigmine seemed to bind to the agonist sites of muscarinic receptors in a competitive manner. Repeated oral administration of distigmine caused a significant decrease in the maximal number of binding sites (B_{max}) for [^{3}H]NMS in the bladder and submaxillary gland but not cerebral cortex. Distigmine also bound to nicotinic receptors in the rat cerebral cortex. In conclusion, distigmine shows direct binding to muscarinic receptors in the rat bladder, and repeated oral administration of distigmine causes downregulation of muscarinic receptors in the rat bladder. The observed direct interaction of distigmine with the bladder muscarinic receptors may partly contribute to the therapeutic and/or side effects seen in the treatment of detrusor underactivity.

Key words detrusor underactivity; distigmine; muscarinic receptor; nicotinic receptor

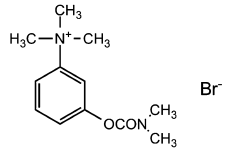
Impaired bladder emptying can be caused by chronic conditions such as bladder outlet obstruction in men with benign prostatic hyperplasia and impaired detrusor contractility in patients of either sex.[1)] Detrusor underactivity is defined as a contraction of reduced strength and/or duration resulting in prolonged bladder emptying within a normal time span.[2)] Some of the established causes of detrusor underactivity include neurogenic, myogenic, aging, and medication side effects.[3)] The medical treatment for detrusor underactivity aims to enhance detrusor contractility and to reduce urethral outlet resistance thereby improving voiding. Standard pharmacotherapy includes the use of α-adrenoceptor antagonists to reduce outlet resistance, muscarinic agonists, and acetylcholinesterase (AChE) inhibitors.

Pharmacotherapy using cholinomimetic drugs such as muscarinic agonists and AChE inhibitors has been used to treat impaired bladder emptying[4,5)] since these drugs improve detrusor contractility by activating the parasympathetic cholinergic system. AChE inhibitors are considered to increase detrusor contractility by inactivating cholinesterase, thus maintaining cholinergic stimulation. Alternatively, the potential interaction of AChE inhibitors with cholinergic receptors has been also implicated in the therapeutic and/or side-effects associated with this class of compounds.[6,7)] It was previously shown that neostigmine interacts directly with muscarinic and nicotinic receptor sites.[6—8)]

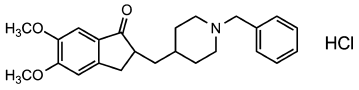
Distigmine bromide (distigmine) (Fig. 1), a reversible and long-acting carbamate cholinesterase inhibitor, has been clinically used to treat patients with voiding dysfunction associated with impaired detrusor contractility.[9—11)] Bougas *et al.*[9)] reported that treatment with distigmine resulted in a statistically significant reduction in residual volume and percentage residual volume, obviating the need for intermittent self-catheterization in patients with detrusor underactivity. In addition, the maximum flow rate and detrusor pressure at maximum flow increased. These results suggested that distigmine has clinical efficacy in patients with detrusor underactivity.[9)] Horinouchi *et al.*[12)] revealed that distigmine significantly potentiated acetylcholine (ACh)-induced contractions of the



Distigmine bromide



Neostigmine bromide



Donepezil hydrochloride

Fig. 1. Chemical Structures of Distigmine Bromide, Neostigmine Bromide, and Donepezil Hydrochloride

* To whom correspondence should be addressed. e-mail: yamada@u-shizuoka-ken.ac.jp

isolated bladder detrusor smooth muscle of guinea-pigs and that it directly contracted the detrusor. However, despite clinical and *in vitro* experimental evidence indicating that distigmine increases detrusor contractility, the precise mechanism underlying the pharmacological effects of this agent including its potential interaction with cholinergic receptors remains to be still clarified.

The present study was undertaken to examine using radioreceptor binding assay whether distigmine binds directly to muscarinic and nicotinic receptors since these receptors play a significant role in the bladder voiding function. The effect of distigmine was also compared with that of neostigmine and donepidil (Fig. 1), clinically used AChE inhibitors.

MATERIALS AND METHODS

Materials [*N*-Methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS, 3030 GBq/mmol), [methyl-^{3}H]oxotremorine-M acetate ([^{3}H]oxotremorine-M, 2804 GBq/mmol), and [5,6-bicycloheptyl-^{3}H]epibatidine ([^{3}H]epibatidine, 2054 GBq/mmol) were purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, U.S.A.). Distigmine bromide (distigmine) was donated by Torii Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were purchased from commercial sources.

Animals Male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) aged 8—10 weeks were used in this study. The rats were housed in the laboratory with free access to food (MF; Oriental East, Tokyo, Japan) and water and maintained under a 12-h dark/light cycle in a room at controlled temperature (24±1 °C) and humidity (55±5%). Then, the rats were exsanguinated by bleeding them from the descending aorta under temporary anesthesia with diethyl ether. The bladder, submaxillary gland and cerebral cortex were then dissected and the tissues were minced with scissors.

Muscarinic Receptor Binding Assay The radioligand-binding assays for muscarinic receptors were performed using the antagonistic radioligand [^{3}H]NMS[13] and the agonistic radioligand [^{3}H]oxotremorine-M,[6,14] as previously described. In the case of the [^{3}H]NMS binding assays, the tissues were homogenized using a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) in 19 volumes of ice-cold 30 mM Na^+/*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer (pH 7.5). The homogenates were then centrifuged at 40000×***g*** for 20 min at 4 °C. The resulting pellets were finally resuspended in ice-cold buffer. For the [^{3}H]oxotremorine-M binding assay, the bladder was homogenized in 19 volumes of ice-cold 20 mM Na^+/HEPES buffer (containing 10 mM $MgCl_2$; pH 7.4). The homogenate was incubated at 37 °C for 30 min and centrifuged at 40000×***g*** for 15 min at 4 °C. The resulting pellet suspension was further centrifuged at 40000×***g*** for 15 min at 4 °C, and the suspension of the resulting pellet mixed with 24 volumes of 20 mM Na^+/HEPES buffer was used. In the displacement experiments, the rat tissue homogenates were incubated with [^{3}H]NMS (0.25 nM) and [^{3}H]oxotremorine-M (0.5 nM) in the presence of various concentrations of drugs. In the saturation isotherm experiments, the rat tissue homogenates were incubated with [^{3}H]NMS (0.06—1.5 nM) and [^{3}H]oxotremorine-M (0.16—5.0 nM) in the absence or presence of concentrations equivalent to the IC_{50} value of each drug. Incubation was carried out for 60 min at 25 °C (in the dark with a sodium lamp in the case of [^{3}H]oxotremorine-M), the reaction was terminated by rapid filtration through Whatman GF/B glass fiber filters (presoaked in 0.05% polyethyleneimine in the case of [^{3}H]oxotremorine-M), and the filters were rinsed with ice-cold buffer. Tissue-bound radioactivity was extracted from the filters by immersion in scintillation fluid, and radioactivity was determined by a liquid scintillation counter. Specific binding was determined experimentally from the difference between counts in the absence and presence of 1 μM atropine.

Nicotinic Receptor Binding Assay The radioligand-binding assays for nicotinic receptors were performed using [^{3}H]epibatidine.[15] The cerebral cortex was homogenized in 19 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4) and the homogenate was centrifuged at 40000×***g*** for 10 min at 4 °C. The resulting pellet suspension was further centrifuged at 40000×***g*** for 10 min at 4 °C, and the suspension of the resulting pellet mixed with 19 volumes of 50 mM Tris–HCl buffer was incubated at room temperature for 1 h. In the inhibition experiments, the homogenate of cerebral cortex was incubated with [^{3}H]epibatidine (0.1 nM) in the presence of various concentrations of AChE inhibitors. Incubation was carried out for 60 min at 25 °C. The reaction was terminated by rapid filtration through Whatman GF/B glass fiber filters presoaked in 0.05% polyethyleneimine, and the filters were rinsed with ice-cold buffer. Tissue-bound radioactivity was extracted from the filters by immersion in scintillation fluid, and radioactivity was determined by a liquid scintillation counter. Specific binding was determined experimentally from the difference between counts in the absence and presence of 30 μM (−)-nicotine hydrogen tartrate.

Measurement of AChE Activity The measurement of AChE activity of whole blood cells with or without AChE inhibitor was carried out according to the method previously reported by Worek *et al.*[16] Briefly, whole blood dilutions were prepared from freshly heparinized blood samples by adding 100 μl to 10 ml ice-cold diluting reagent (0.1 mM phosphate buffer with 0.03% TritonX-100, pH 7.4). After careful mixing, the samples were frozen immediately (−30 °C) and kept until the analysis. For the determination of AChE activity, diluted blood samples (1.5 ml) had 1.5 ml of 100 mM phosphate buffer (pH 7.4) containing 0.32 mM 5,5′-dithio-bid-2-nitrobenzoate (DTNB) and 0.02 mM ethopropadine added to them and were incubated for 10 min at 37 °C with or without AChE inhibitors. Then, 0.05 ml of 28.3 mM acetylthiocholine was added, and the absorbance at 436 nm and 37 °C was measured every 3 min for 9 min, before the reaction velocities were calculated. For the determination of total hemoglobin, 1.4 ml of the blood dilution was mixed with 1.4 ml of the transformation solution (0.3 mM potassium ferricyanide, 0.4 mM potassium cyanide, and 6.0 mM sodium bicarbonate in distilled water and 0.05% (v/v) Triton X-100) and incubated for 10 min at ambient temperature. The absorbance at 546 nm was read and AChE activity was normalized to the hemoglobin content of the sample.

Data Analysis Binding data were subjected to a nonlinear regression analysis using Graph Pad PRISM (ver. 4, Graph Pad Software, San Diego, CA, U.S.A.) as previously noted.[17] The ability of the drugs to inhibit specific binding of radioligand was estimated from their IC_{50} values; *i.e.*, the molar concentration of the unlabeled drug necessary to dis-

place 50% of the specific binding of radioligand. The inhibition constant K_i was calculated from the equation $K_i = IC_{50}/(1+L/K_d)$, where L is the concentration of radioligand. The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) were estimated using the saturation isotherms of each radioligand for the specific binding of [^{3}H]NMS and [^{3}H]oxotremorine-M. Statistical analysis of the data was performed by a one-way analysis of variance (ANOVA), followed by Dunnett's test or Tukey's test for multiple comparisons. Statistical significance was accepted at $p<0.05$.

RESULTS

Inhibition of Acetylcholinesterase Activity Distigmine (3—300 nM), neostigmine (1—100 nM), and donepezil (3—300 nM) inhibited AChE activity in the rat blood in a concentration-dependent manner (Fig. 2). The IC_{50} values for distigmine, neostigmine, and donepezil were 45.0±2.0, 6.9±0.3, and 28.2±1.3 nM, respectively, indicating that neostigmine is a relatively potent inhibitor of AChE.

Muscarinic Receptor Binding Activity in Bladder, Submaxillary Gland and Cerebral Cortex Distigmine (30 nM—30 μM), neostigmine (100 nM—100 μM), and donepezil (100 nM—10 μM) inhibited specific [^{3}H]NMS binding in the bladder (Fig. 3A), submaxillary gland, and cerebral cortex in a concentration-dependent manner. The K_i value for distigmine in the bladder was significantly (2 to 3-fold) lower than those in the submaxillary gland and cerebral cortex (Table 1). The K_i values for distigmine in each tissue were 2 to 3-fold higher than those for donepedil. The K_i values for neostigmine in each tissue were more than 50 μM.

Similarly, distigmine (30 nM—10 μM), neostigmine (100 nM—100 μM), and donepezil (10 nM—10 μM) inhibited specific [^{3}H]oxotremorine-M binding in the bladder (Fig. 3B), submaxillary gland, and cerebral cortex of rats in a concentration-dependent manner. The K_i values for distigmine did not differ significantly among tissues (Table 1). They were roughly similar to those for donepedil and significantly (6 to 48-fold) lower than those for neotigmine. Neostigmine and donepedil showed considerably higher affinity for the [^{3}H]oxotremorine-M binding sites in the cerebral cortex than those in the bladder and submaxillary gland. The ratios of the K_i values of distigmine for [^{3}H]NMS to those for [^{3}H]oxotremorine-M in these tissues ranged form 3.1 to 16 (Table 1). The ratios of the K_i values for donepedil in the bladder, submaxillary gland, and cerebral cortex were smaller than those of distigmine as shown by the values of 1.0, 1.8, and 7.6 respectively. The Hill coefficients for the inhibition of [^{3}H]NMS binding by AChE inhibitors were close to unity and those of [^{3}H]oxotremorine-M were also less than one (Table 1).

Analysis of binding parameters in the presence of each agent at concentrations representing the IC_{50} values for the inhibition of radioligand binding (Fig. 3A) revealed that distigmine (2—7 μM) and donepidil (1—4 μM) had significantly (2 to 3-fold) increased K_d values for specific (+)-[^{3}H]NMS binding in the bladder, submaxillary gland, and cerebral cortex of rats, compared with the corresponding control values (Table 2). A significant (15—26%) decrease in the B_{max} values for specific (+)-[^{3}H]NMS binding was also observed in the bladder and cerebral cortex but not the submaxillary gland of rats. Similarly, distigmine (0.2—0.8 μM), neostigmine (1.0, 30 μM) and donepidil (0.1, 1.0 μM) showed the significantly (2 to 4-fold) increased K_d values for [^{3}H]oxotremorine-M binding in each tissue of the rats, without a sig-

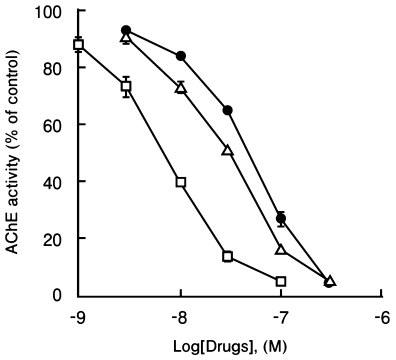


Fig. 2. Effects of Acetylcholinesterase (AChE) Inhibitors on the Rat Blood AChE Activity

The rat blood samples were incubated with different concentrations of distigmine (●), neostigmine (□), and donepezil (△) as described in Materials and Methods. Each point represents the mean±S.E. for three to five experiments.

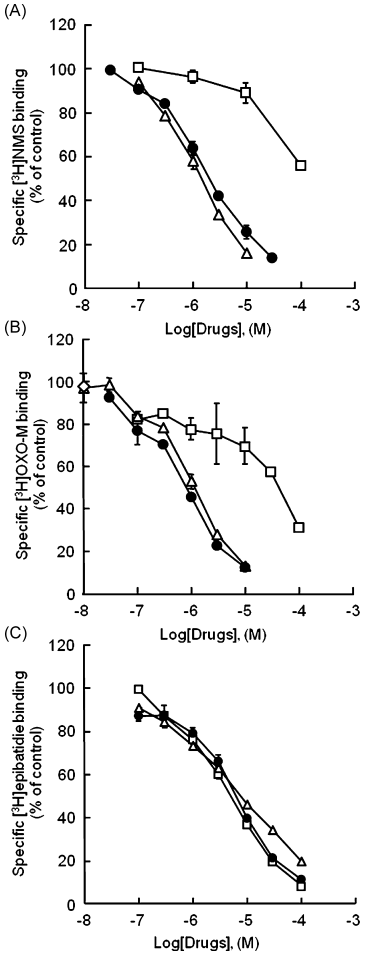


Fig. 3. Competitive Inhibition by Distigmine, Neostigmine, and Donepezil of Specific Binding of [^{3}H]NMS (A), [^{3}H]Oxotremorine (OXO)-M (B), and [^{3}H]Epibatidine (C) in Homogenates of the Rat Bladder

The specific binding of each radioligand in the bladder homogenates was measured in the presence of various concentrations of distigmine (●), neostigmine (□), and donepezil (△). Each point represents the mean±S.E. of three to five experiments.

Table 1. K_i Values and Hill Slopes for the *in Vitro* Inhibition of Specific Binding of [^{3}H]NMS and [^{3}H]Oxotremorine (OXO)-M with Distigmine, Neostigmine, and Donepedil in the Bladder, Submaxillary Gland, and Cerebral Cortex of Rats

	Drugs	K_i (μM)			Hill slopes	
		[^{3}H]NMS	[^{3}H]OXO-M	[^{3}H]NMS / [^{3}H]OXO-M	[^{3}H]NMS	[^{3}H]OXO-M
Bladder	Distigmine	1.13±0.11	0.36±0.12††	3.1	0.88±0.04	0.67±0.13
	Neostigmine	>50	17.4±5.4		—	0.51±0.18
	Donepezil	0.68±0.05	0.70±0.25	1.0	0.88±0.02	0.65±0.03
Submaxillary gland	Distigmine	2.92±0.23*	0.51±0.16††	5.7	0.92±0.03	0.58±0.06
	Neostigmine	>50	20.9±4.9		—	0.53±0.09
	Donepezil	1.63±0.04*	0.91±0.19††	1.8	0.91±0.04	0.73±0.14
Cerebral cortex	Distigmine	2.66±0.29*	0.17±0.09††	16	0.81±0.03	0.77±0.08
	Neostigmine	>50	1.03±0.25*		—	0.49±0.05
	Donepezil	0.84±0.07	0.11±0.02††	7.6	0.92±0.04	0.55±0.05

Rat tissues were subjected to binding assays with [^{3}H]NMS (0.25 nM) and [^{3}H]oxotremorine-M (0.5 nM). Each value represents the mean±S.E. for three to four experiments. Asterisks show a significant difference from the value in the bladder for each drug, $*p<0.05$. Daggers show a significant difference from the values for neostigmine in each tissue, $^{\dagger\dagger}p<0.01$.

Table 2. K_d and B_{max} Values for [^{3}H]NMS Binding of the Rat Bladder in the Absence or the Presence of AChE Inhibitors

	Drugs	K_d (pM)	B_{max} (fmol/mg protein)
Bladder	Control	276±23	120±6
	Distigmine (2 μM)	571±96 (2.07)**	89±4 (0.74)**
	Donepezil (1 μM)	619±38 (2.24)**	91±10 (0.76)*
Submaxillary gland	Control	178±12	141±5
	Distigmine (7 μM)	525±21 (2.95)**	139±5
	Donepezil (4 μM)	552±41 (3.10)**	137±10
Cerebral cortex	Control	210±15	780±29
	Distigmine (6 μM)	467±19 (2.22)**	618±17 (0.79)**
	Donepezil (2 μM)	527±75 (2.51)**	666±25 (0.85)*

Rat tissues were used to estimate the binding parameters of [^{3}H]NMS (0.06—1.5 nM). Each value represents the mean±S.E. for four to eight experiments. Asterisks show a significant difference from the control values, $*p<0.05$, $**p<0.01$. The values in parentheses represent the ratios of K_d or B_{max} relative to the controls.

Table 3. K_d and B_{max} Values for [^{3}H]Oxotremorine-M Binding of the Rat Bladder in the Absence or the Presence of AChE Inhibitors

	Drugs	K_d (pM)	B_{max} (fmol/mg protein)
Bladder	Control	718±82	74±4
	Distigmine (0.7 μM)	2097±217 (2.92)***	68±5
	Neostigmine (30 μM)	2842±201 (3.96)***	68±6
	Donepezil (1.0 μM)	1878±319 (2.62)**	59±7
Submaxillary gland	Control	1603±120	44±3
	Distigmine (0.8 μM)	2586±220 (1.61)**	37±5
	Neostigmine (30 μM)	3545±253 (2.21)***	39±4
	Donepezil (1.0 μM)	2900±248 (1.81)**	46±2
Cerebral cortex	Control	504±39	180±8
	Distigmine (0.2 μM)	981±127 (1.95)*	170±11
	Neostigmine (1.0 μM)	1319±168 (2.62)**	210±20
	Donepezil (0.1 μM)	1002±139 (1.99)*	194±19

Rat tissues were used to estimate the binding parameters of [^{3}H]oxotremorine-M (0.16—5.0 nM). Each value represents the mean±S.E. for four to eight experiments. Asterisks show a significant difference from the control values, $*p<0.05$, $**p<0.01$, $***p<0.001$. The values in parentheses represent the ratios of K_d relative to the controls.

Table 4. Effect of Repeated Administration of Distigmine on the K_d and B_{max} Values for [^{3}H]NMS Binding in Rat Tissues

	Administration	K_d (pM)	B_{max} (fmol/mg protein)
Bladder	Control	293±23	119±14 (1.0)
	Distigmine	228±23	71±11 (0.60)*
Submaxillary gland	Control	277±41	116±8
	Distigmine	193±29	92±6 (0.79)*
Cerebral cortex	Control	207±7	952±37
	Distigmine	210±10	1024±38

Rats orally received distigmine (1.0 mg/kg) or vehicle (control) twice a day for 14 d. The homogenates of rat tissues were used to estimate [^{3}H]NMS (0.06—1.5 nM) binding parameters using saturation isotherms. Each value represents the mean±S.E. for five experiments. Asterisks show a significant difference from the vehicle value, $*p<0.05$.

nificant effect on the B_{max} values (Table 3).

Nicotinic Receptor Binding in the Cerebral Cortex As shown in Fig. 3C, distigmine, neostigmine, and donepezil (each: 100 nM—100 mM) inhibited specific [^{3}H]epibatidine binding in the rat cerebral cortex in a concentration-dependent manner. The K_i value for distigmine (22.9±3.3 μM) was equivalent to the values for neostigmine (18.8±3.3 μM) and donepezil (30.3±2.1 μM).

Effects of Repeated Treatment with Distigmine on Muscarinic Receptors in the Rat Bladder Following repeated oral administration of distigmine at the dose of 1.0 mg/kg, twice a day for 14 d, blood AChE activity was markedly (52%) reduced. In these rats, there were significant (40% and 21%, respectively) decreases in the B_{max} values for [^{3}H]NMS without a change in the K_d values compared with the corresponding control values, suggesting a change in the receptor density (Table 4). The decrease in B_{max} was significantly greater in the bladder than the submaxillary gland. On the other hand, in the cerebral cortex of the distigmine-treated rats, there was little significant alteration in the muscarinic receptor binding parameters.

DISCUSSION

The major findings of this study are that 1) distigmine directly binds to muscarinic and nicotinic receptors in the rat tissues with higher affinity for the former, 2) donepezil dis-

plays similar receptor binding activities to distigmine while neostigmine has much lower binding affinity for muscarinic receptors, and 3) repeated oral administration of distigmine causes downregulation of muscarinic receptors in the bladder and submaxillary gland but not cerebral cortex of rats.

In the current study, distigmine, neostigmine, and donepezil were confirmed to inhibit AChE activity in the rat blood, and the inhibitory effect of distigmine was not much different from that of donepedil and significantly weaker than that of neostigmine. It was found that distigmine, neostigmine, and donepezil competed for specific [^{3}H]NMS binding sites in the bladder, submaxillary gland, and cerebral cortex of rats, indicating significant binding activity of muscarinic receptors. The binding affinity of distigmine was significantly higher in the bladder than in other tissues (Table 1). The muscarinic receptor binding affinity of distigmine in each tissue was much higher than that of neostigmine. The muscarinic receptor binding activity of AChE inhibitors was also confirmed using [^{3}H]oxotremorine-M, a muscarinic agonist radioligand. In fact, relatively low concentrations of distigmine competed successfully for [^{3}H]oxotremorine-M binding sites in rat tissues. Thus, distigmine displayed significantly higher affinity for the binding sites of [^{3}H]oxotremorine-M compared with those of [^{3}H]NMS as revealed by the large ratios of the K_i values for [^{3}H]NMS to those of [^{3}H]oxotremorine-M (Table 1). Previously, it was shown that muscarinic agonists display high affinity for [^{3}H]oxotremorine-M binding with negligible affinity for [^{3}H]NMS binding; whereas, muscarinic antagonists displaced both [^{3}H]oxotremorine-M and [^{3}H]quinuclidinylbenzilate (QNB) to an equivalent extent.[18] Therefore, based on the QNB/oxotremorine-M affinity ratio, it is possible to predict the antagonist or agonist activities of AChE inhibitors for muscarinic receptors. Neostigmine displaced [^{3}H]oxotremorine-M binding with much higher affinity than [^{3}H]NMS (Table 1), confirming a previous observation by Lockhart *et al.*[6] who found its preferential binding to agonist sites of muscarinic receptors. The capacity of distigmine to displace [^{3}H]oxotremorine-M in preference to [^{3}H]NMS binding predicts that this agent binds with higher affinity to the agonist sites than the antagonist sites of muscarinic receptors as seen for neostigmine. In other words, this implies that distigmine acts as an agonist of muscarinic receptors. This finding may underlie the slight contraction in the isolated urinary bladder detrusor of guinea-pigs induced by distigmine demonstrated by Horinouchi *et al.*[12]

Significant displacement by distigmine of [^{3}H]oxotremorine-M binding seems to be unrelated to increased levels of ACh caused by inhibition of AChE activity since the elimination of endogenous ACh in tissue membranes by successive washing did not alter the affinity of [^{3}H]oxotremorine-M compared with control membranes preparations (data not shown). Distigmine displaced [^{3}H]oxotremorine-M binding in the same sub-micromolar concentration range necessary to inhibit AChE activity. The binding of this agent to [^{3}H]oxotremorine-M-labeled muscarinic receptor sites could result from structural and molecular similarities between the agonist or allosteric binding site of these receptors and the substrate binding site of AChE. It is interesting that donepedil displayed similar affinity to the agonist and antagonist sites of muscarinic receptors in the bladder.

Also, distigmine displayed significant binding activity with nicotinic receptors in the rat cerebral cortex, and its affinities for them were considerably lower than its binding affinity for muscarinic receptors. Similar results were observed for neostigmine and donepedil.

Drug–receptor binding characteristics are generally analyzed by saturation isotherms of radioligand binding.[19] It was shown that distigmine and donepedil had significantly (about two-fold) increased K_d values for specific (+)-[^{3}H]NMS binding in the bladder, submaxillary gland, and cerebral cortex of rats. A significant decrease in the B_{max} values for specific (+)-[^{3}H]NMS binding was also observed in the bladder but not the submaxillary gland of rats. These results indicate that distigmine and donepedil bind to the bladder muscarinic receptor antagonist sites in competitive and noncompetitive manners. Distigmine, neostigmine, and donepedil showed significantly increased K_d values for [^{3}H]oxotremorine-M binding in each tissue of the rats without a change in B_{max}, suggesting a competitive mechanism for their binding with muscarinic agonist sites.

It is known that muscarinic receptors undergo compensatory regulation following significant changes in cholinergic neuronal activity.[20—23] Yamada *et al.*[20,21] found that downregulation of muscarinic agonists occurred in the brain, ileum, and striatum of rats after repeated treatment with an organophosphorus AChE inhibitor, diisopropylfluorophosphate (DFP). In the current study, repeated oral administration of distigmine caused a significant decrease in the B_{max} values for specific [^{3}H]NMS binding in the bladder and submaxillary gland. The downregulation of muscarinic receptors was significantly greater in the former tissue. On the other hand, in the cerebral cortex of these distigmine-treated rats, there was little significant alteration in muscarinic receptor binding parameters, suggesting poor permeability of this agent through the blood-brain barrier. These results imply that distigmine administered orally binds preferentially to muscarinic receptors in the bladder, clinically targeted organ. It is likely that the observed decrease in muscarinic receptor density induced by distigmine reflects downregulation of bladder muscarinic receptors by excessive cholinergic stimulation, which results from chronically low AChE levels. In addition, the involvement of direct stimulation of muscarinic receptors by distigmine is also assumed. This idea may be consistent with the finding of Yamada *et al.*[20,21] who indicated a significant involvement of cholinergic nerves in physiological functions in the urinary bladder. Taken together, the observed direct interaction of distigmine with the bladder muscarinic receptors may contribute partly to the therapeutic and/or side effects seen in the treatment of detrusor underactivity.

Acknowledgments This work was supported in part by a grant from the Smoking Research Foundation.

REFERENCES AND NOTES

1) Javlé P., Jenkins S. A., West C., Parsons K. F., *J. Urol.*, **156**, 1014—1018 (1996).
2) Abrams P., Cardozo L., Fall M., Griffiths D., Rosier P., Ulmsten U., Van Kerrebroeck P., Victor A., Wein A., *Urology*, **61**, 37—44 (2003).
3) Chancellor M. B., Kaufman J., *Urology*, **72**, 966—967 (2008).
4) Yoshimura N., Chancellor M. B., *Rev. Urol.*, **6**, S24—S31 (2004).

5) Taylor J. A., Kuchel G. A., *J. Am. Geriatr. Soc.*, **54**, 1920—1932 (2006).
6) Lockhart B., Closier M., Howard K., Steward C., Lestage, P., *Naunyn Schmiedebergs Arch. Pharmacol.*, **363**, 429—438 (2001).
7) Ward T. R., Ferris D. J., Tilson H. A., Mundy W. R., *Toxicol. Appl. Pharmacol.*, **122**, 300—307 (1993).
8) Nagata K., Chao-Sheng H., Jin-Ho S., Narahashi T., *Brain Res.*, **769**, 211—218 (1997).
9) Bougas D. A., Mitsogiannis I. C., Mitropoulos D. N., Kollaitis G. C., Serafetinides E. N., Giannopoulos A. M., *Int. Urol. Nephrol.*, **36**, 507—512 (2004).
10) Yamanishi T., Yasuda K., Kamai T., Tsujii T., Sakakibara R., Uchiyama T., Yoshida K., *Int. J. Urol.*, **11**, 88—96 (2004).
11) Yeo J., Southwell P., Hindmarsh E., *Med. J. Aust.*, **1**, 116—120 (1973).
12) Horinouchi T., Aoki T., Akiyama R., Ono T., Shibano M., Tanaka Y., Koike K., *Pharmacometrics*, **64**, 45—52 (2003).
13) Oki T., Kimura R., Saito M., Miyagawa I., Yamada, S., *J. Urol.*, **172**, 2059—2064 (2004).
14) Sharif N. A., Williams G. W., DeSantis L. M., *Neurochem. Res.*, **20**, 669—674 (1995).
15) Houghtling R. A., Davila-Gararcia M. I., Kellar K. J., *Mol. Pharmacol.*, **48**, 280—287 (1995).
16) Worek F., Mast U., Kiderlen D., Diepold C., Eyer P., *Clin. Chim. Acta*, **288**, 73—90 (1999).
17) Ito Y., Oyunzul L., Seki M., Oki T., Matsui M., Yamada S., *Br. J. Pharmacol.*, **156**, 1147—1153 (2009).
18) Freedman S. B., Harley E. A., Iversen L. L., *Br. J. Pharmacol.*, **93**, 437—445 (1988).
19) Yamada S., Isogai M., Kagawa Y., Takayanagi N., Hayashi E., Tsuji K., Kosuge T., *Mol. Pharmacol.*, **28**, 120—127 (1985).
20) Yamada S., Isogai M., Okudaira H., Hayashi E., *J. Pharmacol. Exp. Ther.*, **226**, 519—525 (1983).
21) Yamada S., Isogai M., Okudaira H., Hayashi E., *Brain Res.*, **268**, 315—320 (1983).
22) Li B., Duysen E. G., Volpicelli-Daley L. A., Levey A. I., Lockridge O., *Pharmacol. Biochem. Behav.*, **74**, 977—986 (2003).
23) Myslivecek J., Duysen E. G., Lockridge O., *Naunyn Schmiedebergs Arch. Pharmacol.*, **376**, 83—92 (2007).