Muscarinic Receptor Binding of Imidafenacin in the Human Bladder Mucosa and Detrusor and Parotid Gland

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Objectives: The current study aimed to characterize comparatively the binding of imidafenacin to muscarinic receptors in the human bladder mucosa and detrusor muscle and parotid gland.

Methods: The muscarinic receptor in homogenates of human tissues (bladder mucosa and detrusor muscle and parotid gland) was measured using a radioligand binding assay with [N-methyl-3H]scopolamine methyl chloride ([3H]NMS).

Results: Imidafenacin competed with [3H]NMS for binding sites in the bladder mucosa and detrusor muscle and parotid gland, and its affinity was significantly (2.6–8.7 times) higher than that of oxybutynin. Also, the affinity of imidafenacin for muscarinic receptors was approximately two-fold higher in the parotid gland than bladder tissue. The affinity of imidafenacin in the mucosa was similar to that in the detrusor muscle, suggesting that this agent exhibits therapeutic effects by blocking muscarinic receptors in the mucosa as well as detrusor muscle. Scatchard analysis revealed that imidafenacin increased significantly (approximately four-fold) Kd values for [3H]NMS binding in the human detrusor muscle and parotid gland, with little effect on Bmax values. This observation indicates that imidafenacin binds to the muscarinic receptors in human tissues in a competitive and reversible manner.

Conclusion: Imidafenacin binds to muscarinic receptors in the human bladder mucosa and detrusor muscle and parotid gland with high affinity. This agent was considered to exhibit therapeutic effects on the lower urinary tract symptoms due to an overactive bladder by blocking muscarinic receptors in the urothelium as well as detrusor muscle.

Key words detrusor, human bladder mucosa, imidafenacin, muscarinic receptor, parotid gland

1. INTRODUCTION

Overactive bladder (OAB), characterized by frequency, urgency, urge incontinence and nocturia, affects many elderly people.1 Antimuscarinic agents used to treat OAB decrease the contractility of the bladder during micturition by blocking muscarinic receptors in the detrusor muscle to increase the capacity of the bladder and decrease urgency during the storage phase.2 It has been shown that the M3 subtype mediates the direct contractile response to acetylcholine in the urinary bladder. In fact, the contractile response to muscarinic agonists exhibited an M3-type profile in a study of competitive antagonism3 and these contractions were markedly decreased in the urinary bladder of M3 receptor knockout mice.4

The bladder urothelium not only provides a barrier to diffusion but also serves a sensory function and releases signaling molecules such as acetylcholine and adenosine 5′-triphosphate. Although the function of such innervation remains unclear, muscarinic receptors are present on the mucosa (urothelium) as well as detrusor muscle of the urinary bladder.5–7 Therefore, mucosal muscarinic receptors may represent a novel site of action of antimuscarinic agents for the treatment of bladder disorders. In this connection, Kim et al.8 showed that intravesically infused antimuscarinic agents suppressed carbachol-induced bladder overactivity, possibly implying a blockage of muscarinic receptors in bladder-afferent pathways.

Most of the side effects of antimuscarinic treatment in patients with OAB are thought to be caused by significant binding to muscarinic receptors in organs other than the bladder.9–11

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bladder. The dry mouth caused by antimuscarinic agents, for example, is mainly due to a blockade of muscarinic receptors in the salivary gland.

The novel antimuscarinic agent imidafenacin, 4-(2-methyl-1-H-imidazol-1-yl)-2,2-diphenyl butanamide (KRP-197/ONO-8025), is currently being developed for the treatment of OAB in Japan. Imidafenacin is a well-tolerated agent with fewer adverse effects.9–12 It has been reported to have high affinity for M₃ receptors, which play an important role in bladder contraction, to exhibit functional selectivity toward the bladder over the salivary gland, and to have little pharmacological effect on the central nervous system at high doses.13,14 Murakami et al.15 showed that imidafenacin exerted an inhibitory effect on postjunctional as well as prejunctional muscarinic receptors to modulate the release of acetylcholine in human detrusor smooth muscles. Thus, imidafenacin may select the bladder over the salivary gland, reducing the risk of side effects on the central nervous system in the treatment of OAB. Recently, we showed that imidafenacin administered orally distributes predominantly to the bladder and exerts a more selective and longer-lasting effect on the bladder than other organs such as the submaxillary gland.16 Furthermore, the imidafenacin excreted in urine may play an important role in the pharmacokinetic and pharmacological selectivity. The present study aimed to compare the binding characteristics of imidafenacin to pharmacologically relevant muscarinic receptors in the human bladder mucosa and detrusor muscle and parotid gland using a radioligand binding assay.

2. METHODS

2.1. Materials

[N-Methyl-³H]scopolamine methyl chloride ([³H]NMS; 3.03 TBq/mmol) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). Imidafenacin was donated by Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were purchased from commercial sources.

Specimens of detrusor muscle and mucosa were collected from bladder carcinoma patients (n = 7) undergoing open surgery, while specimens of parotid gland were obtained from patients undergoing the surgical excision of a parotid tumor (n = 5). Informed consent was obtained from each patient prior to their operation. All specimens used in the experiments were taken from areas macroscopically free of tumors, immediately frozen, and stored at −80°C. This study was conducted according to guidelines approved by the Ethics Committees of the University of Shizuoka, University of Yamanashi, and Hamamatsu University Medical School.

2.2. Tissue preparation

The tissues were carefully minced with scissors and homogenized with a Kinematica Polytron homogenizer in 19 volumes of ice-cold 30 mM Na⁺/HEPES buffer (pH 7.5). The homogenates were then centrifuged at 40,000 × g for 20 min. The resulting pellets were resuspended in the same buffer for the binding assay.

2.3. Muscarinic receptor binding assay

The binding assay for muscarinic receptors was performed using [³H]NMS as described previously.17 In the saturation binding experiments, the tissue homogenate was incubated with various concentrations (0.1–1.5 nM) of [³H]NMS in 30 mM Na⁺/HEPES buffer (pH 7.5). In the competition binding experiments, the tissue homogenate was incubated with [³H]NMS (1.0 nM) in the presence of each antimuscarinic agent. Incubation was carried out for 60 min at 25°C and the reaction was terminated by rapid filtration (Cell harvester; Brandel Co, Gaithersburg, MD, USA) through Whatman GF/B glass filters. The filters were rinsed three times with 3 mL of ice-cold 50 mM Na⁺/K⁺ phosphate buffer (pH 7.4). Tissue-bound radioactivity was extracted from the filters overnight in scintillation fluid, and the radioactivity was measured with a liquid scintillation counter. Specific [³H]NMS binding was determined experimentally from the difference between counts in the absence and presence of 1 μM atropine. Protein concentrations were measured by the method of Lowry et al.18

2.4. Data analysis

The [³H]NMS binding data was subjected to a non-linear regression analysis using Graph Pad PRISM (version 4, Graph Pad Software, San Diego, CA, USA). The apparent dissociation constant (K_d) and maximal number of binding sites (B_max) for [³H]NMS were estimated. The ability of nonlabeled agents to inhibit specific [³H]NMS binding (1.0 nM) was estimated from the IC50, which is the molar concentration of antimuscarinic agents necessary to displace 50% of specific [³H]NMS binding. The inhibition constant, K_i, was calculated from the equation, K_i = IC50/(1 + L/K_d), where L represents the concentration of [³H]NMS. Statistical analyses of the data were performed with Student’s t-test. The data were expressed as the mean ± standard error. Statistical significance was accepted at P < 0.05.

3. RESULTS

3.1. [³H]NMS binding sites in the human bladder mucosa and detrusor muscle and parotid gland

Specific binding of [³H]NMS at 0.1–1.5 nM in homogenates of human bladder mucosa and detrusor muscle and parotid gland was saturable and of high affinity, and the non-specific binding determined in the presence of 1 μM atropine was extremely low. The non-linear regression analysis revealed a linear plot in these tissues, suggesting a single population of high-affinity binding sites for [³H]NMS. The calculated K_d values for specific [³H]NMS binding in the mucosa and detrusor and parotid gland were 237 ± 49, 260 ± 82 and 210 ± 20 pM, respectively, and the B_max values were 85.3 ± 5.7, 69.8 ± 4.3 and 172 ± 50 fmol/mg protein, respectively (Table 1).
chloride ([3H]NMS) binding in homogenates of human detrusor and inhibition of specific [3H]NMS binding in these tissues and mucosa (Table 2). A similar concentration-dependent fold higher in the parotid gland than in the detrusor was observed for oxybutynin (3–300 nM) (Fig. 1). Thus, Ki values of [3H]NMS binding in the bladder mucosa and detrusor muscle and parotid gland was measured. Each value represents the mean ± SEM for four to six samples.

### 3.2. Inhibition by imidafenacin of specific [3H]NMS binding in the bladder mucosa and detrusor muscle and parotid gland

In the competition-binding experiments, imidafenacin (0.3–100 nM) competed with [3H]NMS for binding sites in the bladder mucosa and detrusor muscle and parotid gland in a concentration-dependent manner (Fig. 1). Based on the Ki values, the muscarinic receptor binding affinity of imidafenacin was approximately two-fold higher in the parotid gland than in the detrusor and mucosa (Table 2). A similar concentration-dependent inhibition of specific [3H]NMS binding in these tissues was observed for oxybutynin (3–300 nM) (Fig. 1). Thus, the affinity for muscarinic receptor of imidafenacin was 5.6 (detrusor), 2.6 (mucosa) and 8.7 (parotid gland)-fold higher than that of oxybutynin as shown by the ratios of Ki values and the difference was statistically significant (Table 2). The Hill coefficients for both agents in these tissues were close to unity.

### 3.3. Effect of imidafenacin on [3H]NMS binding parameters in the detrusor muscle and parotid gland

The muscarinic receptor binding of imidafenacin was further characterized by measuring the binding parameters of [3H]NMS in the detrusor muscle and parotid gland in the presence of this agent at concentrations around the IC50 (5 or 10 nM). Imidafenacin increased significantly (4.1-fold) the Kd values for specific [3H]NMS binding in the detrusor muscle and parotid gland (Table 3). On the other hand, the Bmax values were little affected in the presence of imidafenacin. Similarly, oxybutynin (30 nM) significantly (8.9- and 3.2-fold, respectively) enhanced the Kd values for [3H]NMS binding in the detrusor muscle and parotid gland with little effect on the Bmax values (Table 3).

### 4. DISCUSSION

Muscarinic receptor binding sites of imidafenacin in the human bladder mucosa (urothelium) and detrusor muscle and parotid gland were characterized using a radioligand binding assay with [3H]NMS. [3H]NMS exhibited high-affinity binding to homogenates of the mucosa and detrusor muscle, which was specific and saturable. Both Kd and Bmax values for the specific binding of [3H]NMS in the mucosa were comparable to those for detrusor muscle, suggesting the existence of muscarinic receptors in the human urothelium with a similar affinity and density to those in the detrusor muscle.

Imidafenacin competed with [3H]NMS for binding sites in the human bladder mucosa and detrusor muscle and parotid gland, and its affinity was significantly (2.6–8.7 times) higher than that of oxybutynin. Also, the affinity of imidafenacin for muscarinic receptors was significantly higher in the parotid gland than bladder tissue. A similarly high affinity of imidafenacin for muscarinic receptors in the exocrine gland was also observed in rats. The affinity of this agent in the human mucosa was similar to that in the detrusor muscle, suggesting that imidafenacin exhibits therapeutic effects by blocking muscarinic receptors in the mucosa as well as in the detrusor muscle. Further, since imidafenacin is reportedly excreted in human urine at concentrations that exert pharmacological effects on the bladder, the mucosal muscarinic receptors could be blocked by this agent excreted in urine directly from the bladder’s luminal side as well as the circulating blood. In fact, our recent study showed that imidafenacin instilled intravesically at pharmacological doses bound significantly to muscarinic receptors in the rat bladder. Scatchard analysis revealed that imidafenacin increased significantly Kd values for [3H]NMS binding in the human detrusor muscle and parotid gland, with little effect on Bmax values. This observation may indicate that imidafenacin binds to the muscarinic receptors in human tissues in a competitive and reversible manner.

The salivary gland contains predominantly M3 muscarinic receptors, whereas the bladder contains both the M2 and M3 subtypes, though M2 receptors dominate. Furthermore, our recent study with M1–M3 receptor knockout mice has shown that the M2 subtype is expressed predominantly in the submaxillary gland and moderately in the bladder, whereas the M2 receptor is the major subtype in the bladder. Maruyama et al. suggested that human parotid gland contains predominantly the M3 subtype based on results of [3H]NMS binding assays with human tissue and membranes of CHO-K1 cell lines expressing the human M1–M3 receptor subtypes. Consistent with these results, the predominance of the M3 receptor in the human parotid gland was demonstrated directly by a radioligand binding assay.
using N-(2-chloroethyl)-4-piperidinyl diphenylacetate (4-DAMP mustard), an irreversible inactivating agent of the M₃ subtype (Yoshida et al., unpubl. observ., 2010).

Since imidafenacin exhibited greater selectivity for the M₃ than M₂ subtype in CHO-K1 cells expressing human muscarinic receptors, the high affinity of this drug for muscarinic receptors in the human parotid gland reflects M₁-subtype selectivity. In spite of this, imidafenacin was shown to select the bladder over the exocrine gland by in vivo functional and ex vivo muscarinic receptor binding experiments in rats. ¹⁴,¹⁶ Such in vivo results seem to contradict the M₃-subtype selectivity in vitro, but the in vivo bladder selectivity may result mainly from the pharmacokinetics of this agent.¹⁶

In conclusion, the present study has revealed that imidafenacin binds muscarinic receptors in the human bladder mucosa and detrusor muscle and parotid gland in a competitive and reversible manner. Imidafenacin may exhibit therapeutic effects on the lower urinary tract symptoms due to an OAB by blocking muscarinic receptors in the urothelium as well as detrusor muscle.

**Disclosure**

No conflict of interest.

**REFERENCES**


