Possible Involvement of Muscarinic Receptor Blockade in Mirabegron Therapy for Patients with Overactive Bladder

Shizuo Yamada, Junko Chimoto, Mizuki Shiho, Takashi Okura, Kana Morikawa, Hirokazu Wakuda, and Kazumas Shinozuka

Center for Pharma-Food Research (CPFR), Graduate School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan (S.Y., J.C.); Laboratory of Pharmaceutics, Faculty of Pharma-Science, Teikyo University, Tokyo, Japan (M.S., T.O.); and Department of Pharmacology II, School of Pharmacy and Pharmaceutical Sciences, Mukogawa Women’s University, Nishinomiya, Japan (K.M., H.W., K.S.)

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ABSTRACT

The selective β3-adrenoceptor agonist mirabegron, an established alternative to antimuscarinic therapy for patients with overactive bladder, induces additional effects against receptors, transporters, and hepatic enzymes. The present study aimed to elucidate the effects of mirabegron on muscarinic receptors in the rat bladder using radioligand binding and functional assays. Mirabegron (0.1–100 μM) inhibited specific [N-methyl-3H]scopolamine methyl chloride binding in the bladder and other tissues of rats in a concentration-dependent manner. Binding affinity in the bladder was similar to that in the heart and significantly higher than those in the submaxillary gland and brain. Mirabegron induced the concentration-dependent relaxation of carbachol-induced contractions in the rat isolated bladder. Further analyses using a two-site model revealed that the relative quantities of high- and low-affinity components for mirabegron were 44.5% and 55.5%, respectively. Respective pEC50 values were 7.06 and 4.97. Based on the receptor binding affinity and pharmacokinetics of mirabegron, muscarinic receptor occupancy in the human bladder for 24 hours after the administration of a single oral dose of 50 mg mirabegron was 37%–76%. The present results demonstrate for the first time that mirabegron may relax the detrusor smooth muscle not only by β3-adrenoceptor activation but also muscarinic receptor blockade.

SIGNIFICANCE STATEMENT

Mirabegron, the first selective β3-adrenoceptor agonist, represents an alternative to antimuscarinic agents for management of overactive bladder (OAB). The present study aimed to clarify whether mirabegron directly binds to muscarinic receptors and affects cholinergic agonist–induced contractions in rat urinary bladder and to predict muscarinic receptor occupancy in human bladder after oral administration of mirabegron. The results demonstrated that mirabegron therapy for patients with OAB may be due not only to β3-adrenoceptor activation but also muscarinic receptor blockade.

Introduction

Mirabegron (Fig. 1) is the first selective β3-adrenoceptor agonist to be developed for the treatment of patients with overactive bladder syndrome (Takasu et al., 2007; Chapple et al., 2014; Yamaguchi et al., 2019). It induces the β3-adrenoceptor–mediated relaxation of the detrusor smooth muscle in humans and rodents (Takasu et al., 2007; Svalø et al., 2013; Michel and Korstanje, 2016). Therefore, it may be administered instead of antimuscarinic agents to manage overactive bladder (OAB), for which antimuscarinics were found to be unsuitable because of adverse effects, including dry mouth, constipation, somnolence, drowsiness, and blurred vision (Deeks, 2018; Yamada et al., 2018). The off-target effects of mirabegron and its metabolites may be useful in therapeutic strategies for OAB (Dehvari et al., 2018). This agent has been shown to induce adverse effects against a number of related receptors, transporters, and hepatic enzymes (Takusagawa et al., 2012a,b; Alexandre et al., 2016; Mo et al., 2017; Groen-Wijnberg et al., 2017; Dehvari et al., 2018). With the exception of the effects of mirabegron on cardiac β1-adrenoceptors (Mo et al., 2017), which limit its administration to patients with heart conditions, the clinical significance of its off-target effects has not yet been elucidated.

Mirabegron has been shown to decrease acetylcholine release from cholinergic nerves in the urinary bladder of both rats and humans (D’Agostino et al., 2015; Silva et al., 2017). Moreover, previous findings indicated that mirabegron antagonized muscarinic receptors in the bladder (Dehvari et al., 2018), however, the underlying mechanisms currently remain unclear. In material submitted to the Food and Drug Administration by Astellas Co., Ltd., mirabegron was shown to exhibit binding affinity to human M2 muscarinic receptors (Ki value of 2.1 μM) [U.S. Food and Drug Administration. Pharmacology/Toxicology NDA/BLA Review and Evaluation

ABBREVIATIONS: CLr, renal clearance; fu, unbound fraction of mirabegron in plasma; S-HMT, S-hydroxymethyltolterodine; [3H]NMS, [N-methyl-3H]scopolamine methyl chloride; OAB, overactive bladder; UP, rate of urine production pEC50, -log[50% effective concentration:EC50] pIC50, -log[50% inhibitory concentration:IC50].
Materials and Methods

Materials. \[^{[N\text{-Methyl-}{^3}\text{H}]}\text{scopolamine methyl chloride (}{^[3}\text{H}\text{NMS)} (3.03 TBq/mmol), a radioligand that selectively binds to muscarinic receptors, was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). Mirabegron and other chemicals were obtained from commercial sources.

Animals. Eight-to ten-week-old male Sprague-Dawley rats, which were used to measure urodynamic parameters and receptor binding, and 12- to 14-week-old female Wistar rats, which were used to assess contractile responses in isolated bladders, were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rats were housed in the laboratory with free access to food and water and maintained on a 12-hour light/dark cycle in a room with a controlled temperature (23°C) and humidity (55% ± 5%). Experimental protocols received approval from the Committee for Research at the University of Shizuoka and Muko-gawa Women’s University and were performed in accordance with the guidelines for the care and use of laboratory animals of both universities.

Measurements of Muscarinic Receptor Binding Activities in Tissues. Muscarinic receptor binding activities were measured in the bladder and other tissues of rats using a radioligand binding assay with \[^{[3}\text{H}]\text{N-methylscopolamine as a selective radioligand, as previously described (Oki et al., 2005; Maruyama et al., 2006). The bladder, submaxillary gland, heart, and brain (cerebral cortex) were dissected, washed with cold saline, and minced with scissors. Tissue was homogenized with a Kinematica Polytron homogenizer in 19 volumes of ice-cold 30 mM Na\(^+\)/HEPES buffer (pH 7.5). The homogenate obtained was centrifuged at 40,000g at 4°C for 20 minutes. The resulting pellet was reusupended in the same buffer as that for the binding assay. The radioligand binding assay for muscarinic receptors was conducted using \[^{[3}\text{H}\text{NMS}. In the binding assay, tissue homogenates were incubated with \[^{[3}\text{H}\text{NMS (0.10–0.30 nM) in 30 mM Na}\(^+\)/HEPES buffer (pH 7.5) (assay volume: 1 ml) at 25°C for 60 minutes. Rapid filtration (cell harvester; Brandel Co., Gaithersburg, MD) through Whatman glass microfiber B grade (GF/B) filters terminated the reaction, and the filters were rinsed with 3 ml of ice-cold buffer three times. Tissue-bound radioactivity was then extracted from the filters by immersion in scintillation fluid overnight, and radioactivity was assessed using a liquid scintillation counter. Specific \[^{[3}\text{H}\text{NMS binding was calculated experimentally as the difference in counts obtained in the absence (total binding) and presence (nonspecific binding) of 1 µM atropine. The fraction of total binding relative to the amount of radioligand per tube was 0.13–0.20.

Measurement of Contractile Responses in Isolated Bladder Strips. The bladder was removed from each rat under sodium pentobarbital anesthesia (120 mg/kg, i.p.) and immediately placed in oxygenated Krebs’ solution (NaCl 118.4 mM, KCl 4.7 mM, CaCl\(_2\) 2.5 mM, MgCl\(_2\) 1.2 mM, NaHCO\(_3\) 25 mM, NaHPO\(_4\) 1.2 mM, and glucose 11.1 mM) at 37°C. The upper part of the bladder was vertically divided into four parts. The middle third of the detrusor muscle was cut into bladder strips (2 mm wide × 5 mm long), which were mounted in a 10-ml organ bath containing Krebs’ solution at pH 7.4, maintained at 37°C, and continuously gassed with 95% O\(_2\)/5% CO\(_2\). Isometric tension was recorded using a force-displacement transducer (model t-7; NEC San-Ei, Tokyo, Japan) coupled to a dual-channel chart recorder (model 8K21; NEC San-Ei). Strips were placed under a passive tension of 2.94 mN and then equilibrated for 45–60 minutes prior to the initiation of further experiments. The effects of mirabegron on carbachol (3 µM)-induced contractions by isolated rat bladder strips were then investigated. This agent was cumulatively applied to the organ bath after the stimulation with 3 µM carbachol. Data were calculated as a change in values with 100% as the contractile force at 3 µM carbachol in the absence of any agents. The effects of 5-hydroxymethyltolterodine (5-HMT), an antimuscarinic agent, and isoprenaline, a β-adrenoceptor agonist, on carbachol-induced bladder contractions were simultaneously examined as references.

Estimation of Muscarinic Receptor Occupancy by Mirabe-gron in the Human Bladder and Submaxillary Gland from Unbound Plasma and Urine Concentrations. The muscarinic receptor occupancies of mirabegron in the bladder (RO\(_{\text{bladder}}\)) and submaxillary gland (RO\(_{\text{gland}}\)) were estimated based on unbound concentrations in plasma (C\(_{\text{plasma,unbound}}\)) and urine (C\(_{\text{urine,unbound}}\) using the following equations:

$$RO_{\text{bladder}} = \frac{C_{\text{urine,unbound}}}{IC_{50} + C_{\text{urine,unbound}}} \cdot RO_{\text{gland}} = \frac{C_{\text{plasma,unbound}}}{IC_{50} + C_{\text{plasma,unbound}}},$$

in which IC\(_{50}\) is the concentration at which 50% of the receptor binding is inhibited. The fraction of total binding in the human bladder and submaxillary gland may be approximated to C\(_{\text{plasma,unbound}}\) and C\(_{\text{urine,unbound}}\), respectively. C\(_{\text{plasma,unbound}}\) and C\(_{\text{urine,unbound}}\) were calculated using the following equations:

$$C_{\text{plasma,unbound}} = C_{\text{plasma}} \times fu, \quad C_{\text{urine,unbound}} = CL_{\text{u}} \times C_{\text{plasma}} / UP, \quad in which C_{\text{plasma}} is the plasma concentration of mirabegron, fu is the unbound fraction of mirabegron in plasma, CL\(_{\text{u}}\) is the renal clearance of mirabegron, and UP is the rate of urine production (liter per hour). C\(_{\text{plasma}}\) and CL\(_{\text{u}}\) were obtained from a previous study by Krauwinkel et al. (2012) in which 50 mg mirabegron was orally administered to healthy elderly subjects. CL\(_{\text{u}}\), fu, and UP were 8.75 l/h (Krauwinkel et al., 2012), 0.29 (Dickinson et al., 2013), and 0.076 l/h (Yokoyama et al., 2013), respectively.

Data Analysis. The present study was exploratory in the sense of the editorial. Data are shown as mean ± S.D. Competition binding data obtained from experiments using rat tissue membranes were analyzed using the nonlinear regression analysis program GraphPad Prism (Version 7.0; GraphPad Software Inc., San Diego, CA) with a one-site binding model. Data from contractile responses in the isolated rat bladder were fit via nonlinear regression to monophasic or biphasic models using GraphPad Prism. In accordance with the exploratory
nature of the present study, all P values within the manuscript cannot be interpreted as hypothesis testing, only as descriptive.

**Results**

**Binding Activities of Mirabegron to Muscarinic Receptors in the Bladder and Other Tissues of Rats.** Mirabegron (0.1–100 µM) inhibited specific [³H]NMS binding in the bladder (Fig. 2A), submaxillary gland, heart, and brain (cerebral cortex) (Fig. 2B) of rats in a concentration-dependent manner. The pIC₅₀ values of mirabegron for the inhibition of specific [³H]NMS binding were 5.62 ± 0.21 (bladder), 4.61 ± 0.26 (submaxillary gland), 5.69 ± 0.10 (heart), and 4.90 ± 0.09 (brain) (Table 1). The IC₅₀ value of mirabegron in the bladder was similar to that in the heart and approximately 11- and 5-fold lower than those in the submaxillary gland and brain, respectively, indicating the higher affinity of this agent to muscarinic receptors in the bladder and heart than those in the submaxillary gland and brain. The Hill coefficient (slope factor) of mirabegron in each tissue was close to unity, except for in the bladder, in which it was less than one (Table 1).

The antimuscarinic agent 5-HMT (0.3–30 nM) (Maruyama et al., 2006) induced the concentration-dependent inhibition of specific [³H]NMS binding in the rat bladder (Fig. 2A) with a pIC₅₀ value and Hill coefficient of 8.19 ± 0.09 and 1.15 ± 0.60 (n = 4), respectively. The β-adrenoceptor–selective agonist isoprotrotenol (1–100 µM) did not markedly affect specific [³H]NMS binding in the rat bladder (unpublished data).

**Relaxant Responses of Cholinergic Contractility in the Rat Bladder by Mirabegron.** As shown in Fig. 3A, mirabegron at concentrations of 0.1 nM–100 µM caused the concentration-dependent relaxation of carbachol (3 µM)-induced contractions in isolated rat-bladder smooth muscles, and the concentration-response curves of mirabegron were typically shallow. Relaxant responses at low concentrations (0.1 nM–1 µM) of mirabegron were followed by further relaxation at higher concentrations (1–100 µM). An analysis of biphasic relaxant curves using a biphasic model revealed that the relative quantities of high- and low-affinity components for mirabegron were 44.5% and 55.5%, respectively, whereas pEC₅₀ values for these two components were 7.06 and 4.97 (Fig. 3B), respectively.

5-HMT (0.1 nM–100 µM) caused the concentration-dependent relaxation of carbachol (3 µM)-induced contractions in rat isolated-bladder smooth muscle with a pEC₅₀ value of 7.50 (Fig. 3A). Similarly, isoproterenol (0.1 nM–100 µM) relaxed carbachol-induced contractions in a concentration-dependent manner, with a pIC₅₀ value of 6.12.

**Prediction of Muscarinic Receptor Occupancy in Human Tissues by Oral Mirabegron.** A previous study reported that the estimated unbound concentration of mirabegron in plasma reached approximately 20 nM 4 hours after the administration of a single oral dose of 50 mg mirabegron to healthy elderly subjects (Krauwinkel et al., 2012) (Fig. 4A). The CLᵣ of mirabegron (8.75 l/h) was 5-fold higher than its glomerular filtration rate (fu × glomerular filtration rate = 1.7 l/h), indicating that the urine concentration of mirabegron is concentrated in the renal tubules by the tubular secretion and reabsorption of water. Based on these pharmacokinetic parameters, the unbound concentration of mirabegron was estimated to be 1.6–8.2 µM, which was 400-fold higher than its plasma unbound concentration (Fig. 4A). Muscarinic receptor occupancy in the human bladder was estimated from muscarinic receptor binding affinity in the rat bladder and the pharmacokinetic binding parameters of oral mirabegron as described in the Materials and Methods. Predicted receptor occupancy was 37%–76% for 24 hours after the administration of a single oral dose of 50 mg of mirabegron, as shown in Fig. 4B. However, muscarinic receptor occupancy in the human submaxillary gland estimated from the unbound plasma concentration of mirabegron was less than 1%.

**Discussion**

β₂-Adrenoceptor mRNA is predominantly expressed in the human detrusor muscle (Fujimura et al., 1999; Nomiya and

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**Table 1**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>pIC₅₀</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submaxillary gland</td>
<td>4.61 ± 0.26</td>
<td>0.86 ± 0.09</td>
</tr>
<tr>
<td>Bladder</td>
<td>5.62 ± 0.21</td>
<td>0.65 ± 0.06</td>
</tr>
<tr>
<td>Heart</td>
<td>5.69 ± 0.10</td>
<td>0.95 ± 0.07</td>
</tr>
<tr>
<td>Brain</td>
<td>4.90 ± 0.09</td>
<td>0.91 ± 0.06</td>
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Values are the mean ± S.D. of five (bladder) and four (other tissues) measurements.

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**Fig. 2.** Inhibition of specific [³H]NMS binding in rat tissues by mirabegron and 5-HMT [(A) bladder, (B) submaxillary gland (S.gland), heart, and brain]. Muscarinic receptors in rat tissues in the absence and presence of each agent at various concentrations were measured by a radioreceptor binding assay using [³H]NMS as the selective radioligand of muscarinic receptors. The ordinate was shown as percentage of the control-specific binding of [³H]NMS in the absence of any agents. Each point represents the mean ± S.D. of four (5-HMT) and five (mirabegron) measurements for the bladder (A) and four for each tissue (B). M: molar concentration.
Yamaguchi, 2003). The selective β3-adrenoceptor agonist mirabegron is an established alternative to antimuscarinic therapy for patients with OAB. The present study is the first to demonstrate the muscarinic receptor binding affinity of mirabegron in the bladder and other tissues of rats as well as its antagonistic effects on carbachol-induced contractions in the isolated bladder detrusor muscle.

Specific [3H]NMS binding in the bladder, submaxillary gland, heart, and cerebral cortex of rats was suppressed by mirabegron in a concentration-dependent manner. These results provide direct evidence for the first time of the binding of a β3-adrenoceptor agonist to muscarinic receptors in the bladder and other tissues. Five types of muscarinic receptors (M1-M5) have been identified to date, and the M2 and M3 subtypes were previously shown to be strongly expressed in the bladder detrusor muscle (Ito et al., 2009). The M3 receptor has been identified as the primary mediator of detrusor contractions in response to cholinergic activation (Yamanishi et al., 2015). M3 muscarinic receptors were found to be predominantly or exclusively expressed in the salivary gland, such as the submaxillary gland, whereas the M2 and M3 subtypes were both detected in the bladder, with a predominance of the M2 receptor (Wang et al., 1995; Ito et al., 2009; Yoshida et al., 2010). The M1 subtype is predominantly expressed in the cerebral cortex (Oki et al., 2005). The present results revealed that the IC50 values of mirabegron for specific [3H]NMS binding were significantly lower in M2 subtype–dominant bladder and heart than in the M3 subtype–dominant submaxillary gland or M1 subtype–dominant cerebral cortex (Table 1), which provides supportive evidence for the higher affinity of mirabegron for the M2 subtype than for the M3 and M1 subtypes.

Previous studies that investigated the mirabegron-mediated relaxation of the detrusor muscle in humans and rodents used muscle precontracted with carbachol, with pEC50 values of 6.54–5.29 for mirabegron (Takasu et al., 2007; Svalø et al., 2013; Cernecka et al., 2015; Michel and Korstanje, 2016). The EC50 value of mirabegron-induced relaxation in rat bladder strips precontracted with carbachol was 5.1 μM (Takasu et al., 2007). Similar EC50 values have been reported by other studies (Michel and Korstanje, 2016). Although mirabegron-mediated relaxation was detected in rat detrusor muscle precontracted with carbachol or KCl, this response appeared to be stronger in the former (Cernecka et al., 2015).
In the present study, mirabegron at concentrations of 0.1 nM–100 μM caused the concentration-dependent relaxation of carbachol-induced contractions in isolated rat bladder smooth muscles, and the concentration-response curve of mirabegron was shallow (Fig. 3A), which is consistent with previous findings (Takasu et al., 2007; Svalø et al., 2013; Cernecka et al., 2015; Michel and Korstanje, 2016; Dehvari et al., 2018). Cernecka et al. (2015) reported shallow concentration-dependent relaxant curves for mirabegron in the rat and human urinary bladders. Dehvari et al. (2018) obtained similar findings (Hill slope of less than 1) for mirabegron in the detrusor muscle of both humans and rodents in comparisons with isoprenaline-mediated relaxation. Collectively, the present results and previous findings indicate the presence of heterogeneous binding sites for mirabegron or negative cooperativity, suggesting binding to more than one site in the detrusor muscle. The relaxant response observed at low concentrations (0.1 nM–1 μM) of mirabegron appeared to be followed by further relaxation at higher concentrations (1–100 μM). The analysis using the biphasic model revealed that the relative quantities of high- and low-affinity components for mirabegron were 44.5% and 55.5%, respectively (Fig. 3B). Respective EC50 values were 87.3 nM (nanomolar range) and 10.7 μM (micromolar range). The efficacy for the low-affinity component of the relaxant response of carbachol-induced contractions to mirabegron in the rat bladder was consistent with its micromolar binding affinity to competitively inhibit specific [3H]NMS binding by muscarinic receptors in the rat bladder (Table 1). These results indicated the involvement of muscarinic receptors in the relaxation of bladder smooth muscle by relatively high concentrations of mirabegron. Therefore, the efficacy of mirabegron to relax the detrusor muscle may result from not only β3-adrenoceptor activation but also muscarinic receptor blockade.

The clinical significance of the effects of mirabegron on muscarinic receptors in the bladder was investigated in more detail. The pharmacokinetics of mirabegron and the clearance of tissue-bound mirabegron need to be considered. The maximal plasma level of mirabegron in elderly subjects after a single oral dose (50 mg/day) was approximately 85 nM (Krauwinkel et al., 2012). Furthermore, a previous study reported the rapid absorption of mirabegron after its administration to subjects at an oral dose of 50 mg per day for 7 days; time to reach maximum plasma drug concentration (Tmax) was 3 to 4 hours, the terminal plasma half-life was ~60 hours, and ~71% of mirabegron bound to plasma proteins (Krauwinkel et al., 2012). The administration of a single oral dose of [14C]mirabegron to rats was shown to elevate tissue:plasma radioactivity levels in some organs, with ratios increasing to 39%, respectively (unpublished data). Mirabegron is considered to induce pharmacologically relevant muscarinic receptor occupation in the bladder; however, precise estimations of receptor occupancy based on interstitial concentrations are currently limited.

According to the findings of a systematic review and network meta-analysis by Kelleher et al. (2018), combination therapy with solifenacim (5 mg) and mirabegron (25 or 50 mg) was more effective than mirabegron (50 mg) alone but was associated with a higher incidence of anticholinergic adverse effects (dry mouth, constipation, and blurred vision) than solifenacim alone. Herschorn et al. (2017) also reported that the incidence of dry mouth, constipation, and dyspepsia was higher with combination therapy with mirabegron and solifenacim than with monotherapies. Therefore, the higher incidence of anticholinergic adverse events with combination therapy with mirabegron and solifenacim may be attributed to the antagonistic effects of mirabegron on muscarinic receptors in addition to β3 agonist properties. The present results obtained using the rat bladder support the clinical relevance of the blockade of muscarinic receptors by mirabegron.

**Authorship Contributions**

**Participated in research design:** Yamada.  
**Conducted experiments:** Chimoto, Shibo, Morikawa, Wakuda.  
**Performed data analysis:** Okura, Wakuda, Shinozuka.  
**Wrote or contributed to the writing of the manuscript:** Yamada, Okura, Shinozuka.