Pharmacological Characterization of a Novel Beta3 Adrenergic Agonist, Vibegron: Evaluation of Anti-Muscarinic Receptor Selectivity for Combination Therapy for Overactive Bladder.


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Abstract

Although the physiological role of muscarinic receptors in bladder function and the therapeutic efficacy of muscarinic antagonists for the treatment of overactive bladder are well established, the role of β3-adrenergic receptors (β3ARs) and their potential as therapeutics is just emerging. In this manuscript, we characterized the pharmacology of a novel β3AR agonist vibegron (MK-4618, KRP-114V) and explored mechanistic interactions of β3AR agonism and muscarinic antagonism in urinary bladder function. Vibegron is a potent, selective full β3AR agonist across species and it dose-dependently increased bladder capacity, decreased micturition pressure and increased bladder compliance in rhesus monkeys. The relaxation effect of vibegron was enhanced when combined with muscarinic antagonists, but differentially influenced by muscarinic receptor subtype selectivity. The effect was greater when vibegron was co-administered with tolterodine, a non-selective antagonist, compared to co-administration with darifenacin, a selective M3 antagonist. Furthermore, a synergistic effect for bladder strip relaxation was observed with the combination of a β3AR agonist and tolterodine in contrast to simple additivity with darifenacin. To determine expression in rhesus bladder, we employed a novel β3AR agonist probe, [3H]MRL-037, that selectively labels β3 receptors in both urothelium and detrusor smooth muscle. Vibegron administration caused a dose-dependent increase in circulating glycerol and fatty acid levels in rhesus and rat in vivo, suggesting these circulating lipids can be surrogate biomarkers. The translation of our observation to the clinic has yet to be determined, but the combination of β3AR agonists with M2/M3 antimuscarinics has the potential to redefine the standard of care for the pharmacological treatment of overactive bladder.
Introduction

The beta-adrenergic receptor (βAR) family was first described more than 60 years ago (Ahlquist, 1948) and has been since divided into three subtypes: β₁, β₂ and β₃ (Lands et al., 1967; Emorine et al., 1989). For the β₃AR subtype, tissue expression is more restricted compared to β₁ and β₂, with adipose, heart/vasculature, urinary bladder (mRNA) and ovary (protein) believed to have the highest expression (Thomas and Liggett, 1993; Berkowitz et al., 1995; Uhlen et al., 2015). In the urinary bladder detrusor muscle of mammals, all three βAR subtypes mRNA are expressed (Nomiya and Yamaguchi, 2003). Additionally, significant expression of β₃AR protein is observed in bladder urothelium (Limberg et al., 2010), the luminal epithelial lining of the urinary bladder (Birder and de Groat, 2007).

The urinary bladder functions to collect and store urine excreted by the kidneys until voided. Alternating phases of continence and micturition are controlled by the interplay of the central and peripheral nervous system with the local release of regulatory agents (Andersson and Wein, 2004; Beckel and Holstege, 2011). Bladder filling occurs by relaxation of the detrusor muscle (via parasympathetic inhibition) with simultaneous contraction of the urethral sphincters to prevent involuntary emptying. It is also thought that release of norepinephrine from the sympathetic hypogastric nerve resulting in activation of primarily βAR receptors enhances bladder compliance via detrusor relaxation, although only sparse sympathetic innervation is observed in the bladder dome (Fowler et al., 2008). Bladder emptying (so-called micturition) on the other hand occurs via a switch in efferent signaling from sympathetic to parasympathetic, resulting in a release of acetylcholine and to a lesser extent ATP from the pelvic nerve causing detrusor contraction and interruption of release of norepinephrine from the hypogastric nerve.
causing urethral sphincter relaxation. Acetylcholine primarily acts on the detrusor via muscarinic M2 and M3 subtypes while ATP acts on P2X-purine receptors, particularly under pathological conditions (Andersson, 2015), in the detrusor to initiate bladder contraction.

Disruption of this coordinated communication can result in a symptom complex characterized by urinary urgency, with or without urgency-associated urinary incontinence, referred to as overactive bladder (OAB) (Abrams et al., 2002). It is estimated that 12-23% of the general population exhibits symptoms of OAB, with a significant degradation in quality of life (Irwin et al., 2006; Coyne et al., 2011). Management of OAB is typically a multimodal approach, employing both non-pharmacological and pharmacological treatment paradigms. When non-pharmacological approaches (i.e. lifestyle and dietary modification) are ineffective, antimuscarinics are the first-line pharmacological treatment, representing the most commonly prescribed drug therapy (Abrams and Andersson, 2007). Although antimuscarinics have been demonstrated to improve urgency, decrease frequency of micturition and urge incontinence, blockade of the M3 muscarinic receptor can lead to adverse effects (AEs) such as dry mouth and constipation (Abrams and Andersson, 2007). These AEs contribute to a high discontinuation rate resulting in significant unmet medical need for a pharmacological treatment for OAB with an improved AE profile while maintaining or exceeding the efficacy of antimuscarinics (Chapple et al., 2008).

β3AR activation in the bladder represents the most relevant mechanism to increase bladder capacity without affecting bladder contraction. Initial studies in isolated human bladder using non-selective βAR agonists such as isoproterenol demonstrated a pronounced bladder relaxation
(Andersson, 1993). Subsequent studies using selective β3AR agonists in isolated human detrusor muscle strips determined that the observed relaxation was due in to activation of β3AR (Rouget et al., 2014; Gillespie et al., 2015; Michel and Korstanje, 2016). Given increasing evidence for β3AR activation as a treatment for OAB, efforts to discover or repurpose potent and selective β3AR agonists were initiated in recent years (Furuta et al., 2006; Drake, 2008). Subsequently, four selective β3AR agonists, mirabegron (Tyagi and Tyagi, 2010), ritobegron (Maruyama et al., 2012), solabegron (Ohlstein et al., 2012), vibegron (Edmondson et al., 2016) entered clinical trials for treatment of OAB, with published clinical data available for both mirabegron and solabegron. In 2012, mirabegron demonstrated significant efficacy in reducing micturition frequency, urgency incontinence and increasing mean volume per micturition in patients with OAB (Chapple et al., 2014) and subsequently received regulatory approval.

Herein, we describe the pharmacological characterization of the novel β3AR agonist vibegron (MK-4618, KRP-114V) (Edmondson et al., 2016) currently under clinical development for OAB. In addition, we describe the mechanistic interaction of β3AR agonists and antimuscarinics in bladder function and we propose an optimal combination of these mechanisms in the treatment of OAB.
Material and Methods

Subjects
All procedures related to the use of animals were approved by the Institutional Animal Care and Use Committee at Merck Research Laboratories (Rahway, NJ) and conform to the NIH Guide for the Care and Use of Laboratory Animals (8th edition, 2011). A total of 16 adult female rhesus monkeys (Macaca mulatta) weighing 5 to 7 kg and 4-7 years of age were used. Animals were either paired or individually housed in temperature/humidity controlled rooms on a 12-h light/12-h dark cycle and fed standard lab chow (Tekland, Harlan Laboratories, Indianapolis, In.) supplemented with fresh fruit and vegetables with ad libitum water. A total of 81 male adult Sprague-Dawley rats weighing 250 to 350 g (Charles River, Wilmington, MA) were housed in temperature and light-controlled (12-h light/12-h dark cycle) cages, fed standard lab chow (Diet 7012, Harlan) and water ad libitum.

Reagents
Vibegron ((6S)-N-[4-((2S,5R)-5-[(R)-Hydroxy(phenyl)methyl]pyrrolidin-2-yl]-methyl)phenyl]-4-oxo-4,6,7,8-tetrahydropyrrolo[1,2-α]pyrimidine-6-carboxamide), MRL-037 ((R)-2-amino-N-(4-(((2S,5R)-5-(((R)-hydroxy(phenyl)methyl)pyrrolidin-2-yl)methyl)phenyl)-5,6-dihydro-4H-cyclopenta[d]thiazole-4-carboxamide), and [3H] MRL-037 ((R)-2-amino-N-4-(((2S,5R)-5-((R)-hydroxy(phenyl-3,5-t2)methyl)pyrrolidin-2-yl)methyl)phenyl-2,6-t2)-5,6-dihydro-4H-cyclopenta[d]thiazole-4-carboxamide) were synthesized by the Discovery Chemistry Department (Merck Research Laboratories) as previously described (Moyes et al., 2014; Edmondson et al., 2016; Supplemental Figure S1). CL316,243 (disodium 5-[(2R)-2-[(2R)-2-(3-
Chlorophenyl)-2-hydroxyethyl]amino[propyl]-1,3-benzodioxole-2,2-dicarboxylate hydrate), oxybutynin chloride and methoctramine hydrate were purchased from Sigma-Aldrich (St. Louis, MO). Tolterodine L-tartrate and darifenacin hydrobromide were purchased from Toronto Research Chemicals (North York, Canada) and AK Scientific (Union City, CA) respectively.

**Autoradiography of bladder sections with [³H]MRL-037 – ex vivo receptor occupancy assay**

Rhesus monkeys were anesthetized with an intramuscular injection of Telazol (5 mg/kg) or ketamine HCl (20 mg/kg) followed by a lethal dose of a euthanasia drug (i.e. Euthasol; Virbac AH Inc., Fort Worth, TX). The bladder was then surgically removed and cut into 20μm thick sections, collected on superfrost/plus slides using a rapid sectioning cryostat (Leica CM1900) and then air dried for 30-60 min at room temperature. After marking boundaries around the section with pan-pep, the tissue was covered with 600-1000 μl of binding buffer (50 mM Tris-HCl, 2 mM MgCl₂, 1 mM CaCl₂, 5 mM KCl, 0.1% BSA) containing 50 nM of [³H] MRL-037 (specific activity of 30-50 Ci/mmol; Merck Research Laboratories) without or with 10 μM of cold MRL-037 for non-specific binding for 25 min. After incubation, the slides were washed for 4 min with ice-cold binding buffer containing 0.05% TritonX-100 for total of 6 times and then soaked twice into in 30 ml of ice-cold buffer with 0.05% TritonX-100 in a staining jar for 4 min each wash, followed by dipping the slides three times in ice-cold water. Slide were dried and exposed to Kodak MR-2 895 2855 Sigma Kodak® BioMax™ MR film (Cat # Z350400-50EA) for 5 days. Adjacent bladder sections were stained with hematoxylin and eosin for histology following standard protocol (Longnecker, 1966).
In vitro potency and selectivity of vibegron on $\beta$-adrenergic receptors

The ability of vibegron to activate the human, rhesus, rat, and dog $\beta_1$, $\beta_2$ and $\beta_3$AR was measured using CHO cell lines stably expressing the appropriate adrenergic receptor (Candelore et al., 1999). For $\beta_3$AR, the human cell line used expressed $\beta_3$ at levels similar to those observed in human detrusor muscle (Supplemental Figure S2). To quantify the amount of released cAMP following $\beta$-AR activation, the LANCE cAMP kit (Perkin Elmer, Shelton, CT) a time-resolved fluorescence resonance energy transfer immunoassay was used. Compounds were serially diluted in DMSO and an aliquot added to either 384-well or 96-well micro titer plates in assay buffer (5mM HEPES, 0.1% BSA in Hank's Balanced Salt Solution). The reaction was initiated by the addition of 6000 cells per well in assay buffer that also contained a cAMP specific antibody labeled with Alexa Fluor 647 and a phosphodiesterase inhibitor (IBMX, Sigma). In order to examine serum-shifted potency, efficacy was evaluated using an assay buffer containing 40% pooled human, rhesus or rat serum. Because of a smaller assay window with serum compared to the buffer assay, the number of cells was increased to 10,000 per well. Following 30-minute incubation at room temperature, the cells were lysed by the addition of LANCE detection buffer containing a europium-labeled cAMP tracer. Fluorescence was measured following one hour incubation at room temperature using a PE Envision reader, exciting at 340nm and measuring emission at 615 and 665nm. For each assay, a cAMP standard curve was included and used to convert fluorescence readings directly to cAMP amounts. Values were normalized to a known full agonist (isoproterenol) and the EC$_{50}$ and percent maximum activation then determined.

Isometric detrusor muscle tension recordings
Rats were euthanized with CO$_2$ and the entire bladder was surgically removed and the mucosa was left intact. The bladder was then placed in a bath of Krebs solution (113 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 1.2 mM MgSO$_4$, 25 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$ and 1.5 mM glucose). The bladder dome was dissected in 4 approximately equal pieces of about 6 mm x 3 mm. Each strip was placed in a warmed (37°C) organ bath (25 mL) containing oxygenated (95% O$_2$ + 5% CO$_2$) Krebs solution. The strips were tied at one end to the organ bath, and connected at the other end to an isometric force transducer (AD Instruments, Colorado Springs, CO) under a resting tension of 10 mN. The responses of the preparations were recorded by a multiple channel data acquisition system (PowerLab, AD Instruments), and measured with an analysis software (Chart 5, AD Instruments). After the equilibration period for at least 60 min, each tissue strip was challenged to electrical field stimulation (EFS) at 60 Hz; duration, 0.3 ms; 3 sec; 90 V to induce contractions. Upon obtaining stable contractions with EFS, i.e. baseline, compound solution (25 μl) was applied into the organ bath in a cumulative manner, followed by another EFS (consecutive 3 stimulations with 2 min intervals) 15 min after compound treatment. Average amplitude of 3 contractions at baseline and each concentration, changes from baseline and then ratio to corresponding vehicle group (% of control) were calculated.

**Isobologram analysis for assessment of combined drug effects.**

Isobologram analysis (Tallarida, 2001) was used to determine whether the interactions between a selective β$_3$AR agonist CL316,243 and antimuscarinic drugs were additive, synergistic, or antagonistic. CL316,243 was used in this study, as this compound was known to be potent at rat β$_3$AR. Dose-dependent effects were determined for each compound and for combinations, at fixed constant ratios. Data points on the isobologram were evaluated according to their positions.
relative to the diagonal. Data points in the lower left region indicated synergism, falling on the
diagonal line indicated additive effects, and the upper right region indicated antagonism. The
combination index (CI) provided a means to analyze the combined effects with a median-effect
plot analysis. The CI was calculated according to the following formula: CI = (dCL/IC_{25}CL) +
(dA/IC_{25}A), where IC_{25}CL is the concentration of CL316,243 required to produce 25% inhibition
vs. control, and dCL is the concentration of CL316,243 required to produce 25% inhibition when
combined with an antimuscarinic drug. Similarly, IC_{25}A is the concentration of an antimuscarinic
required to produce 25% inhibition, and dA is the concentration of an antimuscarinic required to
produce 25% of the effect when combined with CL316,243. The CI values were defined as
follows: <0.8 = synergism; from 0.8 to 1.2 = additive effect; and >1.2 = antagonism. The IC_{25}
values and their 95% confidence intervals was interpolated from the non-linear fit curve (Prism 5,
GraphPad, La Jolla, CA).

**Cystometry in rhesus monkeys.**

Cystometry was performed as described previously (Nagabukuro et al., 2011). In brief, rhesus
monkeys were anesthetized with an intramuscular injection of Telazol (5 mg/kg) or ketamine
HCl (20 mg/kg) followed by intravenous constant rate infusion with ketamine HCl (0.2-0.3
mg/kg/min). Animals were then placed in a supine position and two catheters (20 gauge) were
inserted into a saphenous and/or a brachial cephalic vein for compound administration and
ketamine infusion/blood sampling, respectively. A triple lumen balloon transurethral catheter
(7.4 Fr, Cook Medical, Bloomington, IN) was inserted into the bladder and the balloon was
inflated with 1 mL of saline to secure the catheter at the bladder base. Another two lumens were
connected to a pressure transducer and an infusion pump for intravesical pressure measurement
and intravesical saline infusion, respectively. The intravesical pressure was recorded using a multiple channel data acquisition system (Power Lab 4/30, AD Instruments, Colorado Springs, CO) at a sampling rate of 20 Hz. After confirming bladder emptiness by ultrasonography, saline was intravesically infused at 15 ml/min. Saline infusion was discontinued when a rapid increase in the intravesical pressure due to the micturition reflex was observed. After two baseline cystometry readings, a compound was intravenously dosed using a rising dose paradigm, with a cystometry measurement performed 10 min after each dose. Blood samples were taken for measurements of plasma compound levels and serum glycerol/free fatty acid (FFA) levels right after each cystometry. The following cystometric parameters were obtained from each cystometry: bladder capacity (duration of bladder filling multiplied by intravesical infusion rate), maximum micturition pressure (the pressure reading of the first peak in intravesical pressure driven by the micturition reflex) and bladder compliance (inverse of average slope of intravesical pressure during filling phase). The changes from baseline were calculated and compared with the values in the vehicle-treated group or baseline values. Compounds were dissolved in sterile 60% PEG400-20% ethanol-20% saline in a volume of 0.2 ml/kg. Serum glycerol levels were determined using commercially available kits (Sigma). Plasma concentrations of compounds were determined by liquid chromatography-tandem mass spectrometry on an Applied Biosystems API 4000 mass spectrometer. Plasma glycerol and FFA levels were determined using commercially available kits (FG0100, Sigma and NEFA-HR2, Wako, respectively) which rely on a series of enzyme coupled reactions.

**Statistical Analysis**
Data are presented as arithmetic mean +/- SD, SEM or geometric mean with confidence intervals (CI) except where indicated. The mean values were compared with two-way analysis of variance (ANOVA) with Bonferroni’s post hoc test or one-way ANOVA with Dunnett’s multiple comparison test. A probability value less than or equal to 0.05 was considered significant.
Results

Potency of vibegron at β Adrenergic receptors across species

Vibegron (Figure 1A) potently activates human β<sub>3</sub>AR and increases cAMP levels, with an EC<sub>50</sub> of 1.1 nM and 87% activation relative to isoproterenol (Edmondson et al., 2016). Vibegron is also highly selective over β<sub>1</sub>AR and β<sub>2</sub>AR versus β<sub>3</sub>AR across multiple species, demonstrating >9000-fold selectivity for activation of β<sub>3</sub>AR over β<sub>1</sub>AR or β<sub>2</sub>AR in cell based in vitro functional assays (Table 1). A small serum shift was observed in the presence of 40% human serum (EC<sub>50</sub> 1.7 nM, Figure 1B). The small effect of serum was also observed for rat and rhesus β<sub>3</sub>AR, consistent with the low non-covalent plasma protein binding (Edmondson et al., 2016).

Localization of β<sub>3</sub>AR in rhesus bladder with a labeled β3AR agonist

In order to determine expression of β<sub>3</sub>AR in rhesus tissue, we used a radiolabeled agonist of β<sub>3</sub>AR, [³H]MRL-037 (Figure 2A; Supplemental Figure S1), a pan-species potent and selective β<sub>3</sub>AR agonist (Moyes et al., 2014), in tissue autoradiography experiments. This compound is a potent β<sub>3</sub>AR agonist across multiple species with no activity at β<sub>1</sub> or β<sub>2</sub> and with physiochemical properties that make it a suitable tracer for localizing β<sub>3</sub>AR expression in tissue. Similar to the staining pattern in human tissue observed with immunohistochemistry (Limberg et al., 2010), clear staining of the urothelium was observed in rhesus bladder using [³H]MRL-037 (Figures 2B and 2C). Staining of human bladder tissue with ³H-MRL-037 produced a similar pattern, albeit with weaker signal than was observed in rhesus (Supplemental Figure S3).

Dose Dependent Effects of Vibegron in urodynamic parameters in rhesus monkeys
We previously reported the activity of vibegron in a rat bladder hyperactivity model (Edmondson et al., 2016), measured by cystometry, and the comparison to activity in adipose tissue as measured by lipolysis readouts. We sought to extend the evaluation of vibegron to non-human primates. A non-human primate pharmacodynamic model offers advantages for pharmacological evaluation of vibegron because of physiological and anatomical similarities of the lower urinary tract to humans. Additionally, vibegron activates both human and rhesus monkey $\beta_3$AR at a similar potency (Table 1). Accordingly, vibegron was evaluated across concentrations up to 10 mg/kg i.v. in the rhesus cystometry model. As investigated in the rat bladder hyperactivity model, a relationship between indices of bladder function and target engagement markers of acute $\beta_3$AR stimulation in adipose tissue, i.e. circulating glycerol increase in rhesus monkeys, was also examined. Baseline values of urodynamic parameters in vehicle- and vibegron-treated animals are as follows: bladder capacity, 156.8 ± 21.6 ml (vehicle), 123.7 ± 10.7 ml (vibegron); micturition pressure, 36.7 ± 1.6 cm H$_2$O (vehicle), 35.5 ± 2.8 cm H$_2$O (vibegron); bladder compliance, 15.0 ± 2.2 ml/cm H$_2$O (vehicle), 16.6 ± 2.8 ml/cm H$_2$O (vibegron). As in the previous study (Nagabukuro et al., 2011), vehicle had no statistically significant effect on any of parameters. Vibegron increased bladder capacity in a dose-dependent manner (Figure 3A). The maximum bladder capacity increase induced by vibegron was 156% of baseline value, which is comparable to maximum effect with antimuscarinics (Nagabukuro, 2011). Micturition pressure was significantly decreased at 0.3 and 3 mg/kg (Figure 3B). Bladder compliance was increased at doses greater than 0.1 mg/kg (Figure 3C). Vibegron also increased serum glycerol and FFA levels in a dose-dependent manner (Figures 3D and 3E). Concentration response curves for bladder capacity and serum glycerol closely overlapped (see Table 2 for vibegron plasma levels), where the EC$_{50}$ value in increasing bladder capacity was 2.9 nM in total.
plasma and 1.5 nM at unbound levels; for increasing serum glycerol, the EC₅₀ value was 9.9 nM in total plasma and 5.0 nM at unbound levels (Edmondson et al., 2016).

**Effect of combined treatments of a β₃AR agonist with antimuscarinics in isolated detrusor muscle.**

CL316,243 and all antimuscarinics oxybutynin, tolterodine and darifenacin, inhibited the EFS-induced isolated detrusor muscle contractions in a concentration-dependent manner (Figure 4A, B). Unlike oxybutynin, vibegron also depressed the spontaneous contractile activity. Concentrations that induced 25% inhibition (IC₂₅) were used to determine combination ratios and for isobologram analyses (Table 3). CL316,243 was co-treated with tolterodine, oxybutynin or darifenacin at a fixed combination ratio. Isobologram analyses are shown in Figure 5. Based on the CI criteria (see Materials and Methods section), all combinations were synergistic, but with different degrees of synergism: tolterodine > oxybutynin > darifenacin (Figure 5). The M₂ selective antagonist methoctramine caused only marginal inhibition of the EFS-induced detrusor contraction (0.003 – 10 μM; Supplemental Figure S4). Given the potency of methoctramine at muscarinic receptor subtypes other than M₂ (Ki, ~1 μM), significant inhibition at the higher concentrations was expected. But at least in the current experimental setting, there were less than 25% inhibition of EFS-induced isolated detrusor contraction. In addition, pretreatment of methoctramine (1 μM) did not affect CL316,243-induced inhibition of detrusor contraction (data not shown). However, methoctramine caused a significant change in isobologram for the darifenacin and CL316,243 combination. As shown in Figure 5D, combination with CL316,243 and darifenacin exhibited much more robust synergism with a pretreatment of methoctramine (CI, 0.16) compared to the same 1 μM).
Effect of combined treatments of vibegron and antimuscarinics in rhesus monkeys

To extend our findings of combination therapy with vibegron, co-treatments of vibegron and two antimuscarinic agents tolterodine and darifenacin were evaluated in the cystometry model in rhesus monkeys. We used monotherapy data for tolterodine and darifenacin from our previous publication (Nagabukuro 2011) as we carried out the study with exactly same methods and within similar timeframe. All dose-combinations of vibegron and tolterodine showed a greater bladder capacity increase compared to each compound alone, with effects that are greater than additive at low doses (Table 4). In contrast, addition of darifenacin to vibegron induced greater bladder relaxation in rhesus only when used at high doses.
Discussion

Given the questionable selectivity of antibodies for staining of β3AR in rhesus monkeys, we turned to a more novel approach of identifying β3AR expression in the urinary bladder of this species – use of a selective β3 agonist radioligand – MRL-037 – to label β3AR expressing cells in the urinary bladder. From our in vitro characterization of MRL-037, this compound is extremely selective for β3 across several species, including rhesus monkeys. Using autoradiography of bladder sections incubated with [3H]MRL-037, β3AR expression was detected in urothelium. While less intense, β3AR expression was detected in detrusor as well, similar to what has been previously observed in human bladder, where β3AR expression appears to be more intense in the urothelium as compared to the detrusor (Limberg et al., 2010). The potency and selectivity of MRL-037 across several species provides for a unique means to directly compare and quantify β3AR expression across multiple species using a single tool. In addition, MRL-037 could be employed to determine ex vivo receptor occupancy, providing a means to directly correlate β3AR target engagement in the bladder to efficacy.

We observed staining for β3AR in the urothelium of human and rhesus monkey. Given our data, along with previously published observations (Limberg et al., 2010; Kullmann et al., 2011; Otsuka et al., 2013), β3AR is expressed in both the detrusor muscle and bladder urothelium across multiple species. The exact role of β3AR in the urothelium and its contribution to the observed efficacy of β3AR agonists in treating OAB is unknown, but there is mounting evidence that β3ARs in the urothelium likely contribute either directly or indirectly to the observed clinical effects of β3AR agonists. Our demonstration of agonist binding to urothelium and the previously
reported β3AR agonist induced functionality in an immortalized human urothelium cell line (Harmon et al., 2005) infer that β3AR agonists can have a direct effect on activating cells within the urothelium. However, the functional role of β3ARs in the urothelium has not been well documented. Masunaga et al. (Masunaga et al., 2010) and Kullmann et al. (Kullmann et al., 2011) showed that in porcine and rat bladder strips relaxation of the detrusor muscle by non-selective βAR and selective β3AR stimulation occurs to the same extent with or without urothelium. Alternatively, others reported that the presence of urothelium diminishes non-selective βAR induced relaxation of isolated human detrusor muscle (Otsuka et al., 2008; Propping et al., 2013). Using subtype selective βAR antagonists, β2AR was suggested to be involved in this urothelial effect, whereas β3AR directly mediates the relaxation of human detrusor, and its involvement did not differ with or without urothelium (Propping et al., 2013).

Medicinal chemistry efforts at optimizing the potency, selectivity and pharmacokinetic properties of a series of pyrrolidine derived amides led to the discovery of vibegron which is currently in late stage clinical trials for OAB (Edmondson et al., 2016). In two different preclinical species, vibegron causes dose-dependent relaxation of urinary bladder, resulting in an increase in bladder capacity and a decrease in micturition pressure. These pharmacodynamic effects in the lower urinary tract accompany an increase in circulating glycerol and FFA, indicating the potential use of β3AR activation in adipose tissue as a surrogate pharmacodynamic/target engagement readout for urinary bladder. Interpretation of the relative pharmacodynamics effects of β3AR activation in adipose tissue to bladder must be done with care when translating across organs and species. In rhesus cystometry studies, elevated levels of serum glycerol correlated with β3AR mediated increases in bladder capacity, with the two
readouts of $\beta_3$AR activation closely overlapping each other. In rats, while decreases in micturition pressure are accompanied by an increase in circulating glycerol levels, we observed elevated glycerol levels in the absence of any effect on bladder compliance suggesting that rat adipose is the more sensitive tissue to $\beta_3$AR activation relative to bladder (Edmondson et al., 2016). While $\beta_3$AR is also involved in lipolysis in human adipose (Bordicchia et al., 2014), the effects of $\beta_3$AR activation in rat adipose appear to be much more profound compared to that observed in human adipose, and this was noted in the early clinical programs looking at $\beta_3$AR agonists for obesity (Ursino et al., 2009). Chronic administration of $\beta_3$AR agonists in obese rats results in weight loss without changes in food intake due to increased energy expenditures (Ursino et al., 2009; Cernecka et al., 2014), but no such effect is observed in humans (Arch, 2008). $\beta_3$AR is the predominant subtype in both white and brown adipose tissue in rats, while in humans and rhesus monkeys only in brown adipose does $\beta_3$AR predominate, with little $\beta_3$AR expression in white adipose (Candelore et al., 1999). This increased expression of $\beta_3$AR in rat adipose and the more pronounced effect on metabolism may explain our observation of differential sensitivity to $\beta_3$AR agonists in rat adipose versus rat bladder. Alternatively, the difference may not be in tissue sensitivity but may reflect physiological differences in the rodent versus primate lower urinary tract such that $\beta_3$AR agonists may play more active of a role in adipose tissue in rats versus primates.

Our studies combining a $\beta_3$AR agonist with antimuscarinics resulted in enhanced bladder relaxation in both rats and rhesus monkeys, with greater synergism when both muscarinic M2 and M3 subtypes were blocked compared with selective blockade of the M3 subtype. Although M3 appears to be the predominant muscarinic subtype in mediating contractile responses in
bladder detrusor muscle, the role of M2 receptors in bladder is less certain despite it being the more highly expressed subtype (>90% in rat bladder) (Wang et al., 1995). It has been suggested that the M2 subtype contributes to bladder relaxation but in a more indirect role through the enhancement of M3 mediated contractions and through inhibition of bladder relaxation (Ehlert et al., 2005; Matsumoto et al., 2012). The inhibition of bladder relaxation mediated by M2 activation is thought to occur via an inhibition of adenylyl cyclase (the M2 receptor subtype is Gi-coupled) which opposes the increase in cAMP elicited by activation of \( \beta_3 \text{AR} \) (Matsui et al., 2003; Ehlert et al., 2007). In the absence of an M2 response, forskolin and isoproterenol exhibit a greater relaxant activity compared to conditions under which M2 is active (Matsui et al., 2003; Ehlert et al., 2007). Inhibition of M2 activity would have a direct impact on the effects of \( \beta_3 \text{AR} \) activation by increasing cAMP levels and would therefore provide greater relaxation mediated through \( \beta_3 \text{AR} \) activation, which is in accordance with our findings in this study. Recently, Furuta et al. (Furuta et al., 2016) reported that in conscious female rats, the combination therapy of a \( \beta_3 \text{AR} \) agonist and muscarinic M3 antagonists was more effective in increasing bladder capacity than monotherapy and that M2 antagonism had no impact on the effect of a \( \beta_3 \text{AR} \) agonist. We also confirmed that simple M2 antagonism did not influence \( \beta_3 \text{AR} \) agonist-induced relaxation of rat bladder strips. Additionally, muscarinic receptor subtype contribution to bladder function may differ between species. In non-human primates, muscarinic subtypes other than M3 appear to contribute more to bladder storage functions than rodents (Nagabukuro et al., 2011). Multiple clinical studies have demonstrated additional benefits by combining solifenacin and mirabegron (Abrams et al., 2015; Kosilov et al., 2015). Since solifenacin is more M3 selective compared to tolterodine and oxybutynin (Ohtake et al., 2007), it may be possible to further improve efficacy.
at bladder relaxation based on our data suggesting a β3AR agonist combined with a non-selective M2/M3 antagonist as the optimal combination therapy in humans.

In conclusion, we have described the pharmacology of a new potent and selective β3AR agonist, vibegron, and mechanistic interplay between β3AR agonists and muscarinic antagonists in urinary bladder. We have also demonstrated that circulating glycerol and free fatty acid levels could potentially be used as surrogate pharmacodynamic readouts to predict bladder effects of vibegron although care must be exercised when comparing pharmacodynamic effects in different tissue beds (Morgan et al., 2012; Cook et al., 2014). The potential for β3AR agonists as monotherapy to effectively treat OAB has been validated with the recent approval of mirabegron, but given that both muscarinic and β3ARs play a critical role in bladder function, additional efficacy (with potential for an improved adverse effects profile) may be achieved by combining standard of care antimuscarinics with β3AR agonists. Our observations indicate that combination of β3AR agonists with dual M2 and M3 antagonists rather than selective M3 antagonists provides optimal efficacy in the treatment of OAB. Our hypothetical translation to the clinic has yet to be determined, but we propose this potential therapeutic approach to redefine the standard of care for the pharmacological treatment of OAB.
Acknowledgments
Author Contributions


Conducted Experiments: Di Salvo, Nagabukuro, Wickham, Abbadie, Fitzmaurice, Gichuru, Kulick, Donnelly, Jochnowitz, Hurley, Pereira Sanfiz, Veronin, Villa Zamlynny, Zycband


Wrote or contributed to the writing of the manuscript: Di Salvo, Nagabukuro, Edmondson, Struthers
References

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Footnotes

1 J. Di Salvo and H. Nagabukuro contributed equally to this publication

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Figure Legends

Figure 1. (A) Structure of vibegron; (B) Activation of recombinant human β3AR by vibegron in the absence (filled circle) or the presence (solid circle) of 40% human serum assayed in a recombinant CHO cell line that stably expresses human β3AR. Percent activity is an average of N=3 determinations; error bars represent standard deviation. Potency of vibegron (1.3 nM) is only slightly less potent (1.7 nM) when assayed in the presence of 40% human serum.

Figure 2. MRL β3AR Agonist [3H] MRL-037 (A) is a potent full agonist at the human (EC50 = 0.12 nM), rhesus (EC50 = 0.12 nM), rat (EC50 = 1.0 nM) and dog (EC50 = 0.19 nM) β3ARs with no affinity for the β1 and β2 subtypes (EC50 and IC50 >10uM all species). Comparison of binding of 3H-MRL-037 to histological staining in rhesus bladder tissue: (B) Autoradiography following incubation of bladder with 50nM [3H]MRL-037; (C) H&E staining; (D) To determine non-specific binding, incubation of bladder with 50nM [3H]MRL-037 and a cold excess (10uM) of unlabeled MRL-037 was performed followed by autoradiography. Arrows indicate regions of intact urothelium. Adjacent sections of bladder are shown. Data are representative of 3 studies.

Figure 3. Effect of vibegron on the bladder capacity (A), maximum intravesical pressure (B), bladder compliance (C), serum glycerol (D) and non-esterified free fatty acids (NEFFA) (E) in anesthetized rhesus monkeys. Values represent mean ± SD of value or of percentage of baseline values as indicated. N=6. *P<0.05, **P<0.01 vs. baseline values, repeated measures ANOVA; #P<0.05, ##P<0.01, vs. vehicle-treated group, Dunnett's multiple comparison test. a - not applicable for statistical analyses because of lack of data from one animal (N=5).
Figure 4. Effects of concentrations of a β3AR agonist CL316,243 and muscarinic antagonists on EFS-induced contraction of rat bladder strips. (A) representative recordings of isometric tension of bladder strips during the course of cumulative treatment of vehicle, CL316,243 and oxybutynin. (B) Dose titration of CL316,243, tolterodine, oxybutynin and darifenacin. N=7-8.

Figure 5 Isobologram analysis of the effects of combined treatments with a β3AR agonist CL316,234 and three different muscarinic antagonists in isolated rat detrusor muscle: tolterodine (A), oxybutynin (B) and darifenacin (C). An additional analysis was done with darifenacin with a pretreatment of methoctramine (D). In parenthesis indicates the combination ratio of two drugs. Each represents mean ± 95% confidence interval.
Tables

Table 1. Potency and selectivity of vibegron at human, rhesus, rat, and dog $\beta_1$, $\beta_2$ and $\beta_3$AR.

Potency was measured using CHO cell lines stably expressing the appropriate receptor (Bmax 50-100 fmols/mg for $\beta_3$AR cell lines, 300-500 fmols/mg for $\beta_1$ and $\beta_2$). To determine potency in the presence of serum, compounds were assayed in the presence of 40% autologous serum (pooled donors). EC$_{50}$ for vibegron in the presence of 40% dog serum was not determined. Activation was determined relative to the known full agonist isoproterenol (1 $\mu$M). EC$_{50}$ of isoproterenol at the human, rhesus monkey, rat and dog $\beta_3$AR receptors was 28 nM, 12nM, 124 nM and 264 nM respectively. Values represent geometric mean with confidence intervals (CI); $N \geq 6$ for all values. $\beta_3$AR potency has been previously described (Edmondson et al., 2016).

<table>
<thead>
<tr>
<th>$\beta$AR Subtype</th>
<th>Species</th>
<th>EC$_{50}$, nM (CI); % activation</th>
<th>EC$_{50}$, nM w/ 40% Serum (CI); % activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_3$</td>
<td>Human</td>
<td>1.0 (1.5,0.71); 84%</td>
<td>1.5 (2.3, 1.0); 102%</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>Human</td>
<td>$&gt;10000$; 5%</td>
<td></td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>Human</td>
<td>$&gt;10000$; 7%</td>
<td></td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>Rhesus Monkey</td>
<td>0.52 (0.81, 0.30); 108%</td>
<td>5.5 (12, 2.6); 98%</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>Rhesus Monkey</td>
<td>$&gt;10000$; 4%</td>
<td></td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>Rhesus Monkey</td>
<td>$&gt;10000$; 0%</td>
<td></td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>Rat</td>
<td>81 (119,55); 83%</td>
<td>118 (161,85); 89%</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>Rat</td>
<td>$&gt;10000$; 0%</td>
<td></td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>Rat</td>
<td>$&gt;10000$; 1%</td>
<td></td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>Dog</td>
<td>10 (15, 7.1); 82%</td>
<td></td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>Dog</td>
<td>$&gt;10000$; 2%</td>
<td></td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>Dog</td>
<td>$&gt;10000$; 1%</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Plasma levels of vibegron in anesthetized rhesus monkeys. Values represent Mean ± SD (N=4-6).

<table>
<thead>
<tr>
<th>Plasma vibegron level (nM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.003 mg/kg</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>0.01 mg/kg</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>0.03 mg/kg</td>
<td>3.0 ± 0.9</td>
</tr>
<tr>
<td>0.1 mg/kg</td>
<td>13.1 ± 3.6</td>
</tr>
<tr>
<td>0.3 mg/kg</td>
<td>43.4 ± 15.4</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>128.5 ± 27.9</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>553.6 ± 144.0</td>
</tr>
</tbody>
</table>
Table 3. Potency of CL316,243, tolterodine, oxybutynin and darifenacin in inhibiting EFS-induced contraction of rat bladder strips and combination index of each antimuscarinic with CL316,243.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC&lt;sub&gt;25&lt;/sub&gt; (nM)</th>
<th>upper 95% CI</th>
<th>lower 95% CI</th>
<th>Combination index with CL316,243</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL316,243</td>
<td>2.86</td>
<td>5.91</td>
<td>1.18</td>
<td>-</td>
</tr>
<tr>
<td>Tolterodine</td>
<td>4.71</td>
<td>11.68</td>
<td>1.89</td>
<td>0.21</td>
</tr>
<tr>
<td>Oxybutynin</td>
<td>22.28</td>
<td>68.45</td>
<td>8.86</td>
<td>0.50</td>
</tr>
<tr>
<td>Darifenacin</td>
<td>5.84</td>
<td>11.29</td>
<td>3.07</td>
<td>0.71</td>
</tr>
</tbody>
</table>
Table 4. Bladder capacity increases with combined treatments with vibegron and tolterodine and darifenacin in rhesus monkeys. Values in table represent Mean ± SEM of % increase over baseline (N=4).

<table>
<thead>
<tr>
<th></th>
<th>vibegron (mg/kg)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>0.003</td>
<td>0.01</td>
<td>0.03</td>
<td>0.1</td>
<td>0.3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.1 ± 3.5</td>
<td>18.0 ± 5.7</td>
<td>27.3 ± 5.1</td>
<td>33.9 ± 9.5</td>
<td>31.3 ± 5.0</td>
<td>55.9 ± 18.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tolterodine (mg/kg)</td>
<td>0.01</td>
<td>10.7 ± 5.0 a</td>
<td>26.7 ± 4.9</td>
<td>31.0 ± 6.3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.03</td>
<td>16.8 ± 13.9 a</td>
<td>35.5 ± 9.3</td>
<td>36.4 ± 4.0</td>
<td>43.4 ± 6.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>40.3 ± 9.8 a</td>
<td></td>
<td>62.9 ± 8.3</td>
<td>56.8 ± 8.1</td>
<td>69.6 ± 14.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Darifenacin (mg/kg)</td>
<td>0.01</td>
<td>8.9 ± 7.1 a</td>
<td>13.7 ± 4.0</td>
<td>23.7 ± 3.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>18.3 ± 8.6 a</td>
<td></td>
<td>37.1 ± 9.4</td>
<td>43.3 ± 12.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>29.4 ± 8.9 a</td>
<td></td>
<td>58.0 ± 19.5</td>
<td>68.7 ± 20.9</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a Data from previous publication (Nagabukuro, 2011)
Figure 5

A

Tolterodine (nM) vs. CL316,243 (nM)

- CL + Tol (1:2)

B

Oxybutynin (nM) vs. CL316,243 (nM)

- CL + Oxy (1:10)

C

Darifenacin (nM) vs. CL316,243 (nM)

- CL + Dar (1:2)

D

Darifenacin (nM) vs. CL316,243 (nM)

- CL + Dar (1:2)
Supplemental Data

Pharmacological Characterization of a Novel Beta3 Adrenergic Agonist, Vibegron: Evaluation of Anti-Muscarinic Receptor Selectivity for Combination Therapy for Overactive Bladder.


Discovery and Preclinical Sciences
Merck Research Laboratories
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Kenilworth, New Jersey 07033 United States

Journal of Pharmacology and Experimental Therapeutics
A

\[
\begin{align*}
X &= H, \text{MRL-037} \\
X &= T, [\text{^3H}] \text{MRL-037}
\end{align*}
\]

**In Vitro** Characterization of MRL-037

<table>
<thead>
<tr>
<th>h/rh IC\textsubscript{50} (nM)</th>
<th>h/rh EC\textsubscript{50} \textsuperscript{a} (nM) (%Act)\textsuperscript{b}</th>
<th>(\beta_3) EC\textsubscript{50} \textsuperscript{a} (nM) (%Act)\textsuperscript{b}</th>
<th>(\beta_3) EC\textsubscript{50} \textsuperscript{a} (nM) + 40% serum (%Act)\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta_1)</td>
<td>(\beta_2)</td>
<td>(\beta_1)</td>
<td>(\beta_2)</td>
</tr>
<tr>
<td>&gt;20,000</td>
<td>&gt;20,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Unless otherwise noted, the results are shown as the geometric mean (n \(\geq\) 2). \textsuperscript{b} % Activation defined as the fitted maximal value of the test compound concentration response expressed as a percent of the maximal response to 1 \(\mu\text{M}\) R-(-)-isoproterenol. \textsuperscript{c} n = 1.

B

**Figure S1.** (A) \(\beta_3\)AR Agonist MRL-037 is a potent and selective full \(\beta_3\) agonist across multiple species. (B) Saturation binding using membranes prepared from CHO human recombinant \(\beta_3\)AR stable cell line (see Candelore et al., 1999 for binding assay methodology). Kd of 4.0nM (N=2) was determined from the saturation studies. Additionally, Vibegron displaces MRL-037 binding, indicating that both Vibegron and MRL-037 most likely binds to the same site on \(\beta_3\).
Figure S2 Determination of β₃AR density in female human normal and incontinent bladder homogenates. Human normal and incontinent bladder tissue (Analytical Biological Services Inc., Wilmington, DE) was homogenized using a Polytron homogenizer (3x 20 second pulses; Kinematica, Lucerne Switzerland) in TME buffer (75 mM Tris, pH 7.4, 12.5 mM MgCl₂, 1.5 mM EDTA) containing a protease inhibitor cocktail (Sigma) at 4°C. The homogenate was then spun for 15 minutes at 3000xg, the pellet was then re-suspended in TME buffer, re-homogenized and re-spun as described above. The supernatants from the two spins were combined and then centrifuged at 17,000xg for 45 minutes. The supernatant was discarded and the pellet resuspended in TME buffer at a protein concentration of approximately 5 mg/ml (as determined using a Bradford Protein Assay and BSA s standard). The ¹²⁵I-CYP binding assay was
performed as described (Candelore et al., 1999) using 80 μg homogenate per point. The assay was incubated at room temperature with shaking until the reaction has reached equilibrium (1 hour).

(A) Binding of $^{125}$I-CYP to human bladder homogenates. Data is mean ± standard deviation of n=4 replicates; assay represents N=1. Displacement of bound $^{125}$I-CYP by cold CYP and the non-selective βAR antagonist MRL-A is equivalent, while ~90% of total CYP binding is displaced by the selective β$_3$AR antagonist MRL-B indicating that ~90% of βAR in human bladder is the β$_3$ subtype. Normal and Incontinent tissue gave similar results. (B) Determination of $^{125}$I-CYP binding and affinity in normal and incontinent human bladder homogenates. Kd and Bmax determined from saturation binding: Normal Bladder=1025 pM, 155 fmoles/mg; Incontinent Bladder=580 pM, 100 fmoles/mg.
Figure S3 Comparison of binding of $^3$H-MRL-037 to histological staining in female human bladder tissue: (A) Autoradiography following incubation of human bladder with 50nM $[^3$H] MRL-037; (B) H&E staining; (C) To determine non-specific binding, incubation of bladder with 50nM $[^3$H]MRL-037 and a cold excess (10uM) of unlabeled MRL-037 was performed followed by autoradiography. Arrows indicate regions of intact urothelium. Adjacent sections of bladder are shown. Data are representative of 4 studies.
**Figure S4** Effects dose titration of Methoctramine on EFS-induced contraction of isolated rat bladder strips. N=12.