Comparative Analysis of the Effects of Antimuscarinic Agents on Bladder Functions in Both Nonhuman Primates and Rodents

Hiroshi Nagabukuro, Katherine L. Villa, L. Alexandra Wickham, Alison A. Kulick, Loise Gichuru, Marcie J. Donnelly, Gregory O. Voronin, Tony Pereira, Xinchun Tong, Andrew Nichols, Stephen E. Alves, Gary P. O’Neill, Christopher V. Johnson, and Emily J. Hickey

Department of Musculo-Skeletal (H.N., S.E.A.), In Vivo Sciences (A.N.), Respiratory and Immunology (G.P.O.), Merck Research Laboratories, Boston, Massachusetts; and Department of Central Pharmacology (K.L.V., L.A.W., L.G., E.J.H.), Laboratory Animal Resources (A.A.K., M.J.D., G.O.V., C.V.J.), Preclinical DMPK (T.P., X.T.), Merck Research Laboratories, Rahway, New Jersey

Received January 21, 2011; accepted March 30, 2011

ABSTRACT

Both the physiological role of muscarinic receptors for bladder function and the therapeutic efficacy of antimuscarinic agents for overactive bladder syndrome are well documented. We investigated the effect of antimuscarinic agents with different subtype selectivity on urodynamic parameters in nonhuman primates and rodents and compared plasma levels of these agents between species. Anesthetized rhesus monkeys were transurethrally catheterized, and the bladder was infused with saline. Urodynamic parameters were measured before and after intravenous drug administration. Tolterodine (nonselective) and oxybutynin (moderately M3-selective) increased bladder capacity at lower doses than those required to decrease micturition pressure. However, higher doses of darifenacin (M3-selective) were needed to increase the bladder capacity than those needed to decrease the micturition pressure. In rats, tolterodine had no effect on the bladder capacity but decreased the micturition pressure at all of the doses administered. Oxybutynin also decreased micturition pressure and increased bladder capacity at the highest dose. Plasma levels of these drugs overlap in both species. These results suggest that, in addition to the M3 receptor, other muscarinic receptor subtypes contribute to regulate bladder storage function in nonhuman primates, since less subtype-selective tolterodine and oxybutynin showed higher specificity to the bladder capacity effect than the effect on micturition pressure compared with M3-selective darifenacin. In addition, the role of muscarinic receptors in bladder storage function varies between primates and rodents. Compared with rodents, muscarinic receptors may play a more active role during the storage phase to regulate the functional bladder capacity in primates.

Introduction

Urinary bladder function is regulated by the sympathetic and parasympathetic nervous systems. Acetylcholine released from parasympathetic nerve endings activates muscarinic receptors on the detrusor muscle, and thereafter induces smooth muscle contraction. Although all five muscarinic receptor subtypes (M1–M5) are expressed in the bladder, the M2 subtype is predominant in many species, including humans (Hegde and Eglen, 1999); however, the M3 receptors play a major role in detrusor contraction (Longhurst et al., 1995; Chess-Williams et al., 2001).

All authors are current or former employees of Merck and Co., Inc., which funded this study.

A part of the results in this study was presented as follows: Nagabukuro H, Villa KL, Voronin GO, Wickham LA, Kulick AA, Gichuru L, Donnelly MJ, Pereira T, Tong X, and Abbadi C (2009) Comparative analysis of the effects of tolterodine on the urodynamic parameters in nonhuman primates and rodents. International Continence Society Annual Meeting; 2009 Oct. 2; San Francisco, CA. International Continence Society, Bristol, UK.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.111.179747.

ABBREVIATIONS: OAB, overactive bladder; ANOVA, analysis of variance.
sensory effect of antimuscarinics is more likely as a mechanism of action because this class of drugs alleviates bladder symptoms, such as the urgency, frequency, and urge urinary incontinence, which occur during the storage phase when parasympathetic efferent activity is normally absent (de Groat et al., 1993).

It is obvious that antagonism of the M3 subtype is crucial for OAB pharmacotherapy because the efficacy of available antimuscarinics is similar, despite differing subtype selectivities against M3. However, there are significant arguments regarding the role of the M2 subtype and additional therapeutic benefits in drugs, which possess M2 antagonism (Abrams and Andersson, 2007; Smith and Wein, 2010). M2 receptors are expressed in the urothelial layer of the bladder, from which non-neuronal mediators are released with bladder distention (Mukerji et al., 2006; Yoshida et al., 2006). Released mediators, such as ATP and acetylcholine, are believed to activate bladder sensory pathways. Recent studies revealed that selective M2 subtype blockade inhibited non-neuronal ATP release (Yoshida et al., 2010) and also suppressed bladder overactivity (Matsumoto et al., 2010).

To measure bladder function, urodynamic studies, which assess bladder storage and voiding function, are commonly used in humans as well as in animals (Abrams, 2005). Bladder capacity is one of the parameters collected in urodynamic studies, and an increase in the bladder capacity is considered to be a preferable effect of OAB therapy, correlating with a decrease in the micturition frequency. Although urodynamic studies are commonly used for diagnostic purposes in clinics and rarely to measure pharmacological treatment outcomes, a few articles have reported the urodynamic effect of antimuscarinics in humans. For example, tolterodine and trospium, both antimuscarinics, increased bladder capacity in patients with detrusor overactivity (Stohrer et al., 1991; Jonas et al., 1997). In addition, in healthy subjects, tolterodine increased the bladder volume, evoking the normal desire to void (Stahl et al., 1995). In contrast, many studies in rats, the most frequently used preclinical species, failed to show an increase in bladder capacity with antimuscarinics (Sasaki et al., 1997; Angelico et al., 2005; Nagabukuro et al., 2010) or showed a bladder capacity increase only at the high doses (Ohtake et al., 2007; Hegde et al., 2009). Despite this paradox, these studies successfully demonstrated the inhibitory effects of antimuscarinics on detrusor contractility by demonstrating a significant decrease in the maximal micturition pressure and a significant increase in the residual urine volume.

There are only a few reports on urodynamic studies in nonhuman primates (Shoukry and Goniem, 1992; Kimura et al., 1997), but one previous study demonstrated that antimuscarinics, including atropine and oxybutynin, dose-dependently increased bladder capacity at the doses that caused a decrease in micturition pressure (Kimura et al., 1997). In light of these cross-species observations, we hypothesized that there could be a species difference in the physiological role of muscarinic receptors with regard to bladder storage function and that the effect of antimuscarinics on bladder capacity might vary between species.

In this study, we investigated the effects of three antimuscarinics with different subtype selectivity (tolterodine, non-selective; oxybutynin, relatively M3-selective; darifenacin, highly M3-selective) (Abrams et al., 2006; Hegde, 2006) on urodynamic parameters in both nonhuman primates and rodents. We also measured plasma levels of the drugs and directly compared the pharmacokinetic/pharmacodynamic relationship between drugs and species.

**Materials 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 Guide for the Care and Use of Laboratory Animals (National Institutes of Health, National Research Council, 1996). The animals were housed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

A total of 12 adult female rhesus monkeys (Macaca mulatta) weighing 5.3 to 6.2 kg (4–7 years of age) were used. The subjects were either paired or individually housed on a 12-h light/12-h dark cycle (lights on at 7:00 AM). Their diet consisted of 2050 Toklad (Harlan Laboratories, Indianapolis, IN) supplemented with fresh fruit and vegetables. Water was freely available. All animals were observed daily by a veterinary technical staff and caretakers for signs of ill health. Subjects were repeatedly used with ≥13-day resting period.

A total of 54 adult female Sprague-Dawley rats weighing 170 to 240 g (Charles River, Wilmington, MA) were housed in a temperature- and light-controlled (12-h light/dark cycle) room and were allowed access to food (Diet 7012; Harlan) and water ad libitum.

**Cystometry in Rhesus Monkeys.** Monkeys were anesthetized with an intramuscular injection of either telazoi (3–5 mg/kg) or ketamine (10–20 mg/kg) followed by intravenous constant rate infusion with ketamine (0.2–0.8 mg/kg/min) using a syringe pump (Medfusion 2010; Medex, Carlsbad, CA). Ketamine was selected because this anesthetic minimally affected urodynamic parameters (Ghoniem et al., 1996). Animals were placed in a supine position, and a triple lumen balloon transurethral catheter (7.4 Fr; Cook Medical, Bloomington, IN) was aseptically inserted into the bladder and the balloon was inflated with 1 ml of water to secure the tip of catheter at the bladder base. The catheter was connected to an infusion pump (Gemini PC-2TX; ALARIS Medical Systems, San Diego, CA) for bladder filling and to a pressure transducer for intravesical pressure monitoring. Intravesical pressure was continuously recorded using a multiple channel data acquisition system (Powerlab, Biopac systems; AD Instruments, Colorado Springs, CO) at a sampling rate of 20 Hz. After confirming bladder emptiness by ultrasonography (Logiq e vet; GE Medical Systems, Waukesha, WI), saline was intravesically infused at 15 ml/min. When the steep rise in pressure, indicative that the micturition reflex was observed (volume threshold), intravesical infusion was stopped and the bladder was manually drained through the urethral catheter using a syringe. Because the balloon restricts the flow of urine passing through urethra, the bladder was rapidly evacuated within approximately 15 to 30 s immediately after the first peak pressure was identified to avoid bladder overdistension. As a result of the rapid bladder evacuation, micturition via the urethra was not observed in most animals.

After two baseline cystometry readings, a drug was intravenously administered three to four times using a rising dose paradigm with a cystometry measurement 10 min after each dose. In the control group, animals were dosed with vehicle in the same manner. Blood samples (0.5 ml) were collected immediately following each cystometric measurement for pharmacokinetic analysis. Heart rate and blood pressure were monitored during the experiment using a lead II ECG and a blood pressure cuff, respectively. The heart rate and blood pressure criteria for discontinuation of compound dosing were defined as >190 bpm and <40 mm Hg, respectively. However, none of the compounds tested in this study induced cardiovascular effects that exceeded the criteria.

**Cystometry in Rats.** Cystometry was performed as described previously (Nagabukuro et al., 2010). In brief, animals were anes-
The effects of tolterodine, oxybutynin, and darifenacin were quantified using cystometry in rhesus monkeys. The drugs were administered intravenously, and the effects on bladder function were measured. The results showed that darifenacin increased bladder compliance and decreased micturition pressure, while oxybutynin decreased micturition pressure. No significant changes were observed in bladder capacity or threshold pressure with any of the drugs.

### Results

**Effect of Antimuscarinics in Rhesus Monkeys.** The baseline values for all urodynamic parameters in each group are shown in Table 1. There was no intergroup difference for any parameter (one-way ANOVA). These values are consistent with previous reports (Shoukry and Ghoniem, 1992; Ghoniem et al., 1996). Figure 1 shows representative cystometry records from animals dosed with vehicle and tolterodine. Although repetitive treatment with vehicle did not alter any urodynamic parameters compared with baseline values, tolterodine, administered with a rising dose paradigm, increased bladder capacity and decreased micturition pressure. Tolterodine, darifenacin, and oxybutynin all significantly increased bladder capacity (Fig. 2, A and B). The maximal increases were 40 ± 10, 29 ± 9, and 71 ± 10% of baseline bladder capacity with tolterodine (0.1 mg/kg), darifenacin (0.1 mg/kg), and oxybutynin (1 mg/kg), respectively. These drugs also decreased micturition pressure in a dose-dependent manner (Fig. 2, C and D), but only oxybutynin increased threshold pressure at the highest dose (Table 2). None of the drugs affected the bladder compliance at any of the doses tested.

**Effect of Antimuscarinics in Rats.** Table 3 summarizes the effects of tolterodine and oxybutynin on urodynamic parameters. In contrast to the effect in rhesus monkeys, tolterodine had no effect on the bladder capacity of rats, whereas micturition pressure was significantly decreased at all of the doses tested. Tolterodine did not affect the threshold pressure. Oxybutynin increased bladder capacity and threshold pressure at only the highest dose (10 mg/kg). At this dose, only a small amount of urine was expelled, indicating that urinary retention was developed due to high doses of oxybutynin. Micturition pressure was decreased by oxybutynin.

### Pharmacokinetic/Pharmacodynamic Analysis

Results from the pharmacokinetic analysis are summarized in Table 4. Plasma levels of tolterodine and oxybutynin at tested doses overlapped in the two species. Plasma levels of the 5-hydroxymethyl metabolite of tolterodine were very low in rhesus monkeys, which may be due to the acute intravenous dosing paradigm.

To compare the potencies of three antimuscarinics in increasing bladder capacity and decreasing micturition pressure, the average plasma levels at minimal effective doses for both parameters were calculated as follows (bladder capacity increase/micturition pressure decrease): 0.111 ± 0.030: 0.346 ± 0.065 µM for tolterodine; 0.025 ± 0.001: 0.008 ± 0.001 µM for darifenacin; and 0.065 ± 0.006: 0.224 ± 0.015 µM for oxybutynin. We then calculated the potency ratio of a
bladder capacity increase to a micturition pressure decrease (average minimal plasma level to increase bladder capacity/average minimal plasma level to decrease micturition pressure) for each drug: 0.32 (tolterodine), 3.1 (darifenacin), and 0.29 (oxybutynin). These ratios suggest that both tolterodine and darifenacin were approximately 3-fold more potent in increasing bladder capacity than in decreasing micturition pressure, compared with darifenacin (approximately 3-fold less potent in bladder capacity than micturition pressure).

For tolterodine, the plasma levels that increased bladder capacity in monkeys (approximately 0.1 μM) produced no effect on bladder capacity in rats (Fig. 3, A and C), although micturition pressure was decreased at a similar range of plasma levels (0.1–0.3 μM) in both species (Fig. 3, B and D). Likewise, the plasma levels of oxybutynin that decreased the micturition pressure in monkeys and rats are similar. Although oxybutynin increased the bladder capacity only at very high plasma levels (4 μM) in rats, bladder capacity in monkeys was increased from lower plasma levels of oxybutynin (0.2 μM).

**Discussion**

There were two key observations in this study: the differential effects of antimuscarinics with various subtype selectivity on urodynamic parameters in nonhuman primates and the differential effects of antimuscarinics on the bladder capacity between monkeys and rats. Acute intravenous administration of tolterodine (nonselective), darifenacin (M3-selective), or oxybutynin (moderately M3-selective) significantly increased bladder capacity and decreased micturition pressure in rhesus monkeys. However, tolterodine and oxybutynin were more potent in increasing bladder capacity than in decreasing micturition pressure, compared with darifenacin. In contrast to rhesus monkeys, but as reported previously (Angelico et al., 2005; Ohtake et al., 2007), tolterodine did not increase bladder capacity in rats, and oxybutynin increased bladder capacity only at the highest dose. From the comparison of the plasma levels, there was dissociation in the effect of antimuscarinics on the bladder capacity between the two species, whereas these drugs significantly decreased micturition pressure at a similar range of plasma levels. Because darifenacin is the most M3-selective antagonist in the three antimuscarinics evaluated, the results suggest that other muscarinic receptor subtypes, in addition to the M3 receptor, contribute to regulate bladder storage function in nonhuman primates. The results also suggest a species difference in the role of muscarinic receptors in bladder storage function between primates and rodents.

Whereas muscarinic M3 receptors primarily mediate acetylcholine-induced contractile response of the detrusor muscle (Abrams et al., 2006; Hegde, 2006) and are also suggested to play a role in bladder sensory transmission (Andersson and Yoshida, 2003; De Laet et al., 2006; Yoshimura, 2007; Matsumoto et al., 2010), the involvement of M2 receptors in bladder physiology has been proposed. In the detrusor muscle, M2 receptors indirectly mediate smooth muscle tone by inhibiting β-adrenoceptor-mediated relaxation through cAMP signaling (Hegde et al., 1997). Both M2 and M3 receptors are expressed in the urothelial and suburothelial layers (Mukerji et al., 2006), which are a putative site of action for antimuscarinics based on a receptor binding study using human bladder mucosa (Mansfield et al., 2009). In functional studies, a selective blockade of M2 receptors inhibited the release of non-neuronal ATP from the urothelium (Yoshida et al., 2010) and also inhibited bladder overactivity-induced by intravesical administration of a pan-muscarinic agonist (Matsumoto et al., 2010). In this study, darifenacin, the most M3-selective antagonist, showed less specificity to the increase in bladder capacity relative to its effect on micturition pressure.
pressure (decrease in detrusor contraction) than the other two antagonists tolterodine and oxybutynin in monkeys. Because the latter two antagonists possess more potent M2 antagonism than darifenacin (Abrams et al., 2006; Hegde, 2006), the differential effects of the three antimuscarinics on bladder capacity and micturition pressure may be due to the subtype selectivity of these drugs. Although it was reported that selective M2 antagonists did not increase bladder capacity in normal healthy rats (Kim et al., 2005), this might be due to the species differences in the roles of muscarinic receptors in the bladder, as addressed later.

In addition to M2 and M3 receptors, other muscarinic receptor subtypes, such as M1 and M4, are suggested to play roles in regulating bladder functions. For example, prejunctional M3 receptors facilitate and M4 receptors inhibit acetylcholine release from postganglionic nerve endings (Chapple et al., 2002). Central M1 and M4 receptors also are implicated to

### TABLE 2

Effect of tolterodine, darifenacin, and oxybutynin on threshold pressure and bladder compliance in rhesus monkeys

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Threshold Pressure (Changes from Baseline)</th>
<th>Bladder Compliance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cmH2O</td>
<td>baseline</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Tolterodine</td>
<td></td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Darifenacin</td>
<td>0.003</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Oxybutynin</td>
<td>0.1</td>
<td>1.4 ± 1.1</td>
<td>115 ± 7</td>
</tr>
</tbody>
</table>

**P < 0.01 vs. vehicle, two-way ANOVA with Bonferroni’s post hoc test.

**P < 0.01 vs. vehicle, two-way ANOVA with Bonferroni’s post hoc test.

Fig. 2. Effect of tolterodine, darifenacin, and oxybutynin on the bladder capacity (A and C) and micturition pressure (B and D) in rhesus monkeys. *, P < 0.05 and **, P < 0.01, versus vehicle group, two-way ANOVA with Bonferroni’s post hoc test. Each bar represents mean ± S.E.M.
shown an increase only at the high doses (Sasaki et al., 2007). In contrast, many studies have evaluated antimuscarinics in the urodynamic parameters of the drugs (Suzuki et al., 2005). Our results are consistent with previous observations, but we have tested multiple antimuscarinics with different subtype selectivity and evaluated their pharmacokinetics. These additions further clarify the species differences in the effect of antimuscarinics on bladder capacity.

Pharmacokinetic analysis in this study revealed that the plasma levels of tolterodine and oxybutynin overlapped in the two species, whereas the effects of these drugs on urodynamic parameters differed. Tolterodine obviously increased bladder capacity in monkeys but not in rats, whereas oxybutynin increased bladder capacity in both rhesus and rats but only at the highest doses in rats. It is noteworthy that the plasma levels of tolterodine and oxybutynin, which decreased the micturition pressure, were similar in two species (0.1–0.3 μM for tolterodine; approximately 0.2 μM for oxybutynin). However, there are certain caveats when directly comparing plasma drug levels because of the following reasons. Although all three antimuscarinics are known to highly bind to plasma proteins (96–99%) in humans (Abrams et al., 2006; Hegde, 2006) and circulating free drug levels are generally used to interpret target receptor occupancy, the plasma protein binding of each compound may vary between species. In addition, there may be species differences in the muscarinic receptor antagonistic activity of the drugs, although these differences are expected to be marginal based on previous reports (Ohtake et al., 2007; Sinha et al., 2010). Despite these caveats, as well as existence of active metabolites for tolterodine and solifenacin, increase bladder capacity at reasonable doses from clinical use of these drugs (Suzuki et al., 2005). Our results are consistent with previous observations, but we have tested multiple antimuscarinics with different subtype selectivity and evaluated their pharmacokinetics. These additions further clarify the species differences in the effect of antimuscarinics on bladder capacity.
dose. In addition, in rats, oxybutynin significantly increased bladder capacity at the highest dose (10 mg/kg). The results suggest that oxybutynin affected the bladder capacity via effects other than simply through its antimuscarinic activity, which further modifies bladder sensory transmission. In fact, it is well documented that oxybutynin acts as a local anesthetic at high concentrations (De Wachter and Wyndaele, 2003).

It is possible that the species differences we observed in this study were attributed to the technical differences in the two cystometry procedures. In particular, the definition of micturition pressure was slightly different in the two species because of the distinct intravesical catheterization procedures (transurethral in monkeys and transvesical in rats). This may have resulted in a variable sensitivity to detect a decrease in detrusor contractility with antimuscarinics. In addition, whereas transurethral catheterization in monkeys does not cause bladder damage, transvesical catheter implantation in rats can cause acute local inflammation. Antimuscarinics are known to reduce detrusor overactivity in a pathogenic model of topical and acute application of proinflammatory prostaglandin E_{2} to the urethra (Yokoyama et al., 2007); therefore, the minor inflammation that may have been induced in rats could have altered the effects of antimuscarinics compared with the study in monkeys. In addition, the anesthesia used was different between monkeys (ketamine) and rats (urethane). Because muscarinic receptors in the central nervous system are implicated in modifying micturition reflex (Kono et al., 2006), different anesthetics may result in different central effects of antimuscarinics.

In conclusion, in addition to M3, other muscarinic receptor subtypes may contribute to regulate bladder storage functions in nonhuman primates. Furthermore, the role of muscarinic receptors in bladder storage function varies between primates and rodents. In primates, muscarinic receptors may play a more active role in regulating the functional bladder capacity during the storage phase than in rodents.
Antimuscarinics on Bladder Function in Primates and Rodents

Acknowledgments
We thank Dr. Scott Edmondson (Medicinal Chemistry Department, Merck Research Laboratories) for the synthesis of 5-hydroxyethyl tolterodine.

Authorship Contributions
Participated in research design: Nagabukuro.
Conducted experiments: Nagabukuro, Villa, Wickham, Kulick, Gichuru, Donnelly, Voronin, and Pereira.
Contributed new reagents or analytic tools: Villa, Wickham, Kulick, and Pereira.
Performed data analysis: Nagabukuro, Villa, Pereira, and Tong.
Wrote or contributed to the writing of the manuscript: Nagabukuro, Villa, Wickham, Kulick, Gichuru, Pereira, Nichols, Alves, O’Neill, Johnson, and Hickey.

References

Address correspondence to: Dr. Hiroshi Nagabukuro, Department of Musculo-Skeletal, Merck Research Laboratories, EMM8-126, 33 Avenue Louis Pasteur, Boston, MA 02115. E-mail: hiroshi_nagabukuro@merck.com