Effects of Anticholinergic Drugs Used for the Therapy of Overactive Bladder on P-Glycoprotein Activity

Hirokazu Wakuda,a Takashi Okura,b Kana Maruyama-Fumoto,c Satomi Kagota,c Yoshikiko Ito,2 Shino Miyachi-Wakuda,d Naoyuki Otani,d Naoto Uemura,d Shizuo Yamada,d and Kazumasa Shinozukad

aDepartment of Clinical Pharmacology and Therapeutics, Faculty of Medicine, Oita University; 1–1 Idaigaoka, Hasama-machi, Yufu, Oita 879–5593, Japan; bLaboratory of Pharmaceutics, Faculty of Pharma-Sciences, Teikyo University; 2–11–1 Kaga, Itabashi-ku, Tokyo 173–8605, Japan; cDepartment of Pharmacology, School of Pharmacy and Pharmaceutical Sciences, Mukogawa Women’s University; 11–68 Koshien, Kyuban-cho, Nishinomiya, Hyogo 663–8179, Japan; and d Center for Pharma-Food Research, Graduate School of Pharmaceutical Sciences, University of Shizuoka; 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan.

Received May 17, 2019; accepted September 5, 2019

INTRODUCTION

Overactive bladder (OAB), as defined by the International Continence Society, is a syndrome that is usually characterized by increased daytime urination frequency and nocturia, with or without incontinence.14 OAB occurs in at least 10% of the adult population, and the prevalence increases with age.1516 In patients with OAB, muscarinic receptors associated with bladder contraction may be overactivated, leading to frequent urination and incontinence.

Anticholinergic drugs are the primary treatment for OAB.5,17 Because elderly individuals often take multiple medications, clinicians and pharmacists must pay special attention to potential drug–drug interactions, which may cause adverse events or alter drug efficacy in such patients. Adverse drug reactions in older adults are often attributed to polypharmacy.6

Typical anticholinergic side effects include dry mouth, constipation, somnolence, and blurred vision.7 However, anticholinergic therapy in the elderly is also associated with cognitive impairment,8 and the side-effect profile of some anticholinergic drugs for OAB is consistent with inhibition of muscarinic receptors in the central nervous system.9 The incidence of anticholinergic agent-induced adverse effects on the central nervous system is generally much lower than that of dry mouth.3,17 However, adverse effects on the central nervous system can be of great concern, particularly in the elderly.80 The penetration of the central nervous system by drugs depends on the permeability properties of blood–brain barrier and the influence of active efflux transporters present in brain tissue, such as P-glycoprotein. Therefore, the relative permeability and affinity of OAB agents for P-glycoprotein is an important consideration in understanding their potential to exert adverse effects manifested in the central nervous system.7

The contributing factor to a wide range of drug–drug interactions is P-glycoprotein, an ATPase-coupled drug excretion pump that is constitutively expressed in several human tissues, including the intestinal epithelium and the endothelium of the blood-brain barrier. Numerous drugs have been identified as substrates, inhibitors, or inducers of P-glycoprotein. P-Glycoprotein may contribute to the discrepancy between lipid solubility and the blood-brain barrier permeability, and therefore, it is necessary to investigate interactions with P-glycoprotein when considering the influence of P-glycoprotein on the absorption, distribution, metabolism, and excretion (ADME) of anticholinergic drugs.7

If an anticholinergic drug becomes a P-glycoprotein substrate, drug interactions may occur at the blood-brain barrier and in the intestine, and the subsequent side effects such as dry mouth, cognitive impairment, and altered blood drug levels must be taken into consideration in the case of drug combination. Therefore, it is important to understand the relationship between anticholinergic drugs and P-glycoprotein.

We evaluated the effects of anticholinergic drugs principally used for the therapy of overactive bladder (OAB) on the activity of P-glycoprotein, an efflux transport protein, in Caco-2 cells. The time-dependent changes in the fluorescence of residual rhodamine 123, a P-glycoprotein activity marker, in the apical region of Caco-2 cells were measured in the presence of anticholinergic drugs using time-lapse confocal laser scanning microscopy. The effect of anticholinergic drugs on human P-glycoprotein ATPase activity was also measured. The fluorescence of residual rhodamine 123 in untreated Caco-2 cells decreased over time. The gradual increase in the fluorescence was significantly inhibited by treatment with cyclosporine A, darifenacin, and trospium. In contrast, oxybutynin, N-desethyl-oxybutynin (DEOB), propiverine, and its active metabolites (M-1, M-2), imidafenacin, solifenacin, or tolterodine had little effect on the efflux of rhodamine 123. P-Glycoprotein ATPase activity was increased by darifenacin. Darifenacin and trospium reduced the rhodamine 123 transfer across the apical cell membrane. These data suggest that darifenacin and trospium interact with P-glycoprotein. Additionally, darifenacin influenced P-glycoprotein ATPase activity. These results suggest that darifenacin may be a substrate of P-glycoprotein. This study is the first paper to test simultaneously the effects of 10 anticholinergic drugs used currently for the therapy of OAB, on the P-glycoprotein.

Key words anticholinergic drug; overactive bladder; P-glycoprotein

© 2019 The Pharmaceutical Society of Japan
Recently, we developed a new method to assess P-glycoprotein activity utilizing time-lapse confocal laser scanning microscopy, which has increased sensitivity compared with currently used methods that rely on microplate readers and fluorescence microscopy.1,12 The current study aimed to evaluate the effects of darifenacin, N-desethyl-oxybutynin (DEOB), imidafenac, 1-methyl-4-piperidyl diphenyl-propoxypacetate N-oxide (M-1), 1-methyl-4-piperidyl benzilate N-oxide (M-2), oxybutynin, propiverine, solifenacin, tolterodine, and trospium used for the therapy of OAB, on P-glycoprotein activity using our new time-lapse confocal laser scanning microscopy method. Furthermore, the effects of these agents on the stimulation of P-glycoprotein ATPase activity were examined.

MATERIALS AND METHODS

Drugs and Cells Cycloporsine A, verapamil, oxybutynin chloride (oxybutynin), and trospium chloride (trospium) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Imidafenac was provided by Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan). Tolterodine tartrate (tolterodine) was provided by Pfizer Inc. (New York, NY, U.S.A.). Solifenac succinat (solifenacin) was provided by Astellas Pharma Inc. (Tokyo, Japan). Propiverine hydrochloride (propiverine), M-1, and M-2 were provided by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). DEOB and darifenacin were provided by Professor Shizuo Yamada, Ph.D. (University of Shizuoka). Oxybutynin chloride (oxybutynin), and trospium chloride (trospium) were purchased from DS Pharma Biomedical (Osaka, Japan). Darifenacin and solifenacin were provided by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). Propiverine hydrochloride (propiverine), M-1, and M-2 were provided with 10% fetal bovine serum and 1% nonessential amino acids purchased from DS Pharma Biomedical (Osaka, Japan) and M-2 are active metabolites of propiverine. Trospium is an anticholinergic drug and blocks calcium channels. M-1 and M-2 are competitive antagonists of M1 and M3 muscarinic acetylcholine receptors. DEOB is an active metabolite of oxybutynin. Imidafenac competitively antagonizes M1 and M3 muscarinic acetylcholine receptors. Darifenacin competitively antagonizes M2 and M3 muscarinic acetylcholine receptors. Darifenacin and solifenacin competitively antagonize M3 muscarinic acetylcholine receptors. Propiverine has anticholinergic effects and blocks calcium channels. M-1 and M-2 are active metabolites of propiverine. Trospium is an antispasmodic and anticholinergic drug.

The human colon adenocarcinoma cell line (Caco-2) was purchased from DS Pharma Biomedical (Osaka, Japan) and cultured in minimal essential medium with GlutaMAX™ (Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum and 1% nonessential amino acids in an atmosphere containing 5% CO2 and 95% air at 37°C. The cells were sub-cultured to approximately 80% confluence using 0.02% ethylenediaminetetraacetic acid (EDTA) and 0.25% trypsin. The cells were used for the experiments 10 d after seeding.

Measurement of P-Glycoprotein Activity Using Confocal Laser Scanning Microscopy The P-glycoprotein activity was measured according to the method previously reported.1,12 Transport measurements were performed in a Transwell® chamber (BD Biosciences, San Jose, CA, U.S.A.). Caco-2 cells were seeded on polyethylene terephthalate filters (3 x 104 cells/ filter; pore size, 0.4 µm; BD Biosciences) in cell culture inserts (0.3 cm2 effective growth area). All experiments were performed at 37°C in Hank’s balanced salt solution (HBSS: 137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.4 mM MgSO4, 0.5 mM MgCl2, 0.3 mM Na2HPO4, 0.4 mM KH2PO4, 4.2 mM NaHCO3, 25 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), and 5.6 mM glucose). The cell culture insert was placed on a glass-bottom dish, and the Caco-2 cells were incubated with 5 µM rhodamine 123 for 60 min at 37°C. The cells were rinsed three times with HBSS. The dish containing the cell culture insert was wrapped with parafilm around the edges to prevent water vapor loss and placed onto the heated stage (37°C) of the time-lapse confocal laser scanning microscope (Clsi; Nikon, Tokyo, Japan). The argon laser (85BCD20053; Melles Erriot, Albuquerque, NM, U.S.A.) emitted light at an excitation wavelength of 488 nm. The emitted light was collected through a long-pass dichroic reflector (515 nm) and a long-pass emission filter (515 nm) with a Plan Fluor objective (40× magnification; numerical aperture = 0.6). The cell cultures were equilibrated at 37°C to stabilize the cells and the pericellular environment, after which cycloporsine A, anticholinergic drugs,13,14) or the HBSS vehicle were added (final concentrations: cycloporsine A, 10 µM; darifenacin, 10 or 100 µM; DEOB, 100 µM; imidafenacin, 10 µM; M-1, 100 µM; M-2, 100 µM; oxybutynin, 10 µM; propiverine, 100 µM; solifenacin, 100 µM; tolterodine, 100 µM; trospium, 10 or 100 µM; due to poor solubility, imidafenac and oxybutynin could not be implemented at 100 µM). QUantification of the intracellular rhodamine 123 concentration in the Caco-2 cells was performed using a chronological measurement program (EZ-C1; Nikon) to analyze the fluorescence images at several time points (0, 10, 20, 30, and 60 min).

Twenty cell-slice images were captured along the z-axis at equal intervals from the apical brush-border membrane to the basal membrane with a 12-bit dynamic range. Approximately 75 cells are included in each cell-slice image. One representative cell was chosen randomly from each Transwell® chamber, and an image under the apical membrane was immediately analyzed. The fluorescence intensity at 0 min (the time of test substance or vehicle administration) was defined as 100%.

P-Glycoprotein ATPase Activity The effect of anticholinergic drugs on human P-glycoprotein ATPase activity was determined using a human P-glycoprotein membrane (BD Biosciences, Woburn, MA, U.S.A.) and the ABC transporter ATPase reagent kit (Genomenome, Yokohama, Japan). In brief, human P-glycoprotein membrane (20 µg) suspensions were preincubated at 37°C for 5 min in reaction buffer containing 40 mM Tris-Mops (pH 7.0), 50 mM KCl, 2 mM dithiothreitol, 500 µM ethylene glycol bis(2-aminoethyl ether)-N,N,N,N’-tetraacetic acid (EGTA), 5 mM sodium azide, 1 mM ouabain, and anticholinergic agents (1, 3, 10, 30, or 100 µM), with or without 500 µM sodium orthovanadate. The ATPase reaction was initiated by the addition of 4 mM Mg ATP, and the mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of 10% sodium dodecyl sulfate (SDS) solution, and the amount of inorganic phosphate was immediately determined. The samples were comprised of the supplements and the detection mixture (one-part 35 mM ammonium molybdate in 15 mM zinc acetate and four-parts fresh 10% ascorbic acid). Samples were maintained at 37°C for 20 min. The amount of inorganic phosphate complex was determined by measuring the absorbance at a wavelength of 655 nm using a microplate spectrometer (Bio-Rad Laboratories). Vanadate-sensitive ATP hydrolysis was measured by subtracting the value obtained with the vanadate-co-incubated membrane fractions from that obtained with each of the vanadate-free membrane fractions. Verapamil (1–100 µM) was used as a positive control.13,14 The ATP hydrolysis rate (v) was fitted to the following equation using a nonlinear least-squares
regression analysis using Prism software (GraphPad Software, La Jolla, CA, U.S.A.):

\[ v = \frac{V_{\text{max}} \times s}{K_m + s} \]

where \( s \), \( V_{\text{max}} \), and \( K_m \) represent the test compound concentration (\( \mu \)M), maximum ATP hydrolysis rate (nmol/mg protein/min), and Michaelis constant (\( \mu \)M), respectively.

**Statistical Analysis**

All values are presented as the mean ± standard error of the mean (S.E.M.). The data were analyzed using one-way ANOVA followed by a post hoc Dunnett’s test or two-way ANOVA followed by Bonferroni’s post-test for significant results. \( p \)-Values less than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism 4.03 software (GraphPad Software).

**RESULTS**

The residual fluorescence intensity decreased over time, and this decrease was significantly inhibited by cyclosporine A as a positive control (Fig. 1a). The cells treated with DEOB (100 \( \mu \)M), imidafenacin (10 \( \mu \)M), M-1 (100 \( \mu \)M), M-2 (100 \( \mu \)M), oxybutynin (10 \( \mu \)M), solifenacin (100 \( \mu \)M), toloterol (100 \( \mu \)M), and propiverine (100 \( \mu \)M) exhibited decreases in the fluorescence of rhodamine 123, similar to that observed in vehicle-treated control cells (Table 1). However, the fluorescence levels of cells treated with darifenacin (Figs. 1b, c) and trospium (Figs. 1d, e) were significantly different from control cells. After 20 min, the fluorescence intensity levels of cells treated with 100 \( \mu \)M darifenacin and 100 \( \mu \)M trospium were significantly higher than control cells. Furthermore, after 60 min, the fluorescence intensity levels of the cells treated with 10 \( \mu \)M darifenacin was significantly higher than control cells.

The effects of darifenacin (100 \( \mu \)M) and trospium (100 \( \mu \)M) on human P-glycoprotein ATPase activity were determined using human P-glycoprotein membranes. Trospium did not affect P-glycoprotein ATPase activity, whereas darifenacin enhanced P-glycoprotein ATPase activity (Fig. 2). The ATPase activity of darifenacin was concentration-dependent, as was...
the case with verapamil which was used as positive control (Fig. 3). The kinetic analysis provided a $K_m$ value of 10.95 $\mu$M and a $V_{\text{max}}$ value of 50.30 nmol/min/mg protein for darifenacin and a $K_m$ value of 8.125 $\mu$M and a $V_{\text{max}}$ value of 71.33 nmol/min/mg protein for verapamil.

**DISCUSSION**

Fluorescence levels of residual rhodamine 123, which reflect P-glycoprotein activity, in the apical regions of cells treated with anticholinergic drugs were measured using a novel time-lapse confocal laser scanning microscopy method and compared with control cells. We found that darifenacin and trospium inhibited P-glycoprotein-mediated rhodamine 123 efflux from Caco-2 cells, whereas DEOB, imidafenacin, M-1, M-2, oxybutynin, solifenacin, tolterodine, and propiverine had little effects. Moreover, darifenacin was shown to enhance P-glycoprotein ATPase activity.

Caco-2 cells have been used widely as a cell-based model to study transport and absorption across human intestinal epithelia. Hunter et al. reported the functional expression of P-glycoprotein in the apical membranes of human intestinal Caco-2 cells.\(^{17}\) Rhodamine 123 is a lipophilic cation that is selective for P-glycoprotein-dependent transport\(^{18}\) and has been widely used as an *in vitro* and *in vivo* marker of P-glycoprotein function.\(^{19-22}\) Jouan et al. reported that rhodamine 123 could be used for the prediction of clinical P-glycoprotein inhibition according to the Food and Drug Administration (FDA) criteria, with notable sensitivity (80%).\(^{23}\) For example, the National Cancer Institute of the United States uses rhodamine 123 to screen for drugs that serve as substrates for P-glycoprotein.\(^{24}\) Van der Sandt et al. reported rhodamine 123 is a substrate for both P-glycoprotein and the organic cation carrier systems in the kidney cell line. However, in the Caco-2 cell line, rhodamine 123 is selectively transported by P-glycoprotein.\(^{25}\) Therefore, changes in rhodamine 123 levels in Caco-2 cells reflect P-glycoprotein function. By using time-lapse confocal laser scanning microscopy, the effects of 10 anticholinergic agents on residual fluorescence of rhodamine 123 in the apical region of Caco-2 cells were examined. Cyclosporine A, a potent inhibitor of P-glycoprotein, immediately abolished rhodamine 123 efflux from Caco-2 cells, as measured by the

---

**Table 1. Influence of Anticholinergic Drugs on Residual Rhodamine 123 Fluorescence in the Apical Region of Caco-2 Cells at 60 min**

| Sample                          | Final concentrations (after 60 min) |
|---------------------------------|------------------------------------|
| Darifenacin                     | 10 $\mu$M 1.14 **                  |
|                                 | 100 $\mu$M 1.24 ***                |
| DEOB (Active metabolite of oxybutynin) | 100 $\mu$M 0.99                 |
| Imidafenacin                    | 10 $\mu$M 1.02                    |
| M-1 (Active metabolites of propiverine) | 100 $\mu$M 1.07         |
| M-2 (Active metabolites of propiverine) | 100 $\mu$M 1.03         |
| Oxybutynin                      | 10 $\mu$M 0.99                    |
| Propiverine                     | 100 $\mu$M 1.06                    |
| Solifenacin                     | 100 $\mu$M 1.05                    |
| Tolterodine                     | 100 $\mu$M 0.96                    |
| Trospium                        | 10 $\mu$M 1.18 ***                |
|                                 | 100 $\mu$M 1.34 ***               |

*Final vs. control’ indicates the ratio to control cells. **$p<0.01$, ***$p<0.001$ for the control group vs. the drug-treated group.
decrease in fluorescence. Of the anticholinergic agents, darifenacin and trospium inhibited P-glycoprotein-mediated rhodamine 123 efflux transport from Caco-2 cells, suggesting that darifenacin and trospium interact with P-glycoprotein. Conversely, imidafenacin, oxybutynin, solifenacin, tolterodine, propiverine, and active metabolites of oxybutynin (DEOB) and propiverine (MI, M2) did not interact with the P-glycoprotein-mediated transport. These results suggest that darifenacin and trospium could potentially induce drug interactions due to their inhibition of P-glycoprotein, but other anticholinergic drugs may not induce drug interactions via P-glycoprotein.

Next, the P-glycoprotein effects of darifenacin and trospium on ATPase activity were measured because the substrate activity cannot be evaluated by measuring rhodamine 123 efflux alone. The P-glycoprotein ATPase activity was enhanced by darifenacin, but not trospium. These results suggest that darifenacin is a substrate of P-glycoprotein, a finding in line with a previous report whereas trospium has little detectable P-glycoprotein ATPase activity. The kinetic analysis for concentration-dependent P-glycoprotein ATPase activity showed that \( K_m \) and \( V_{max} \) values for darifenacin were similar with values for verapamil, suggesting that darifenacin potentially interacts with P-glycoprotein. Indeed, darifenacin was previously shown to potentially affect P-glycoprotein, because darifenacin changed the blood concentration of digoxin, a well-characterized P-glycoprotein substrate, in humans. Further, darifenacin has low intracerebral transferability despite high lipophilicity. Like verapamil, the potent P-glycoprotein ATPase activity of darifenacin can contribute to the P-glycoprotein-mediated efflux transport of this drug across the blood-brain barrier. The \( K_m \) value of 10.9 \( \mu \)M is higher than its \( K_m \) value for muscarinic receptor binding, suggesting that the P-glycoprotein-mediated transport of darifenacin should be unsaturated at its pharmacologically relevant concentration. In contrast, trospium has low lipophilicity, low intracerebral transferability, and no detectable P-glycoprotein ATPase activity in this study. Taking these findings into consideration, it is suggested that low lipophilicity is one of the main factors for its low intracerebral transferability. Additionally, Sandage et al. reported that trospium did not affect in a clinically meaningful manner the pharmacokinetics of co-administered digoxin in humans.

One of the causes of those differences may be the difference in lipophilicity. Darifenacin has high lipophilicity, while trospium has low lipophilicity. Furthermore, Mukhametov et al. reported that P-glycoprotein has two docking areas and showed that intermediate compounds and high affinity substrates might behave differently in relation to the P-glycoprotein. Therefore, it is posited that darifenacin and trospium may have different P-glycoprotein binding sites as one of the causes. The mechanism of this behavior needs to be considered in the future.

Clinically, this study is useful for avoiding potential P-glycoprotein-related drug interactions, and for choosing first-line therapy. The anticholinergic drugs for OAB in common use are all metabolized through different mechanisms. Therefore, the risk of enhanced drug effects is increased when potentially interacting substrates compete for the same metabolic pathways. Additionally, in a geriatric population or those with neurological or cognitive impairment, it is best to avoid drugs that can cross the blood–brain barrier. Considering this study, darifenacin and trospium are the preferred first-line therapies for patients who are concerned about brain side-effects. The limitation of this study is that it is challenging to clarify the lipophilicity and the differences in binding sites using this method. This point requires further study.

In conclusion, the present study using a novel time-lapse confocal laser scanning microscopy method showed that darifenacin and trospium interacted with P-glycoprotein, whereas DEOB, imidafenacin, M-1, M-2, oxybutynin, solifenacin, tolterodine, and propiverine did not. Moreover, it was shown that darifenacin might be a P-glycoprotein substrate, which could explain its low intracerebral transferability, despite its high lipid solubility. Further, in vivo and clinical studies are necessary to investigate interactions between anticholinergic drugs administered for OAB and P-glycoprotein to enable safe and effective administration of these compounds.

Acknowledgments The authors are very grateful to Ms. Maki Asano, Ms. Michie Uno, Ms. Yurie Kamatsuki, and Ms. Maya Hamazato, for their experimental assistance.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Haylen BT, de Ridder D, Freeman RM, Swift SE, Berghmans B, Lee J, Monga A, Petri E, Rizk DE, Sand PK, Schaer GN. An International Urogynecological Association (IUGA)/International Continence Society (ICS) joint report on the terminology for female pelvic floor dysfunction. Neurourol. Urodyn., 29, 4–20 (2010).
2) Temml C, Haidinger G, Schmidbauer J, Schatzl G, Madersbacher S. Urinary incontinence in both sexes: prevalence rates and impact on quality of life and sexual life. Neurourol. Urodyn., 19, 259–271 (2000).
3) Wilson I, Abrams P, Cardozo L, Roberts RG, Thüroff JW, Wein AJ. How widespread are the symptoms of an overactive bladder, and how are they managed? A population-based prevalence study. BJU Int., 87, 760–766 (2001).
4) Chapple CR, Khullar V, Gabriel Z, Dooley JA. The effects of antimuscarinic treatments in overactive bladder: a systematic review and meta-analysis. Eur. Urol., 48, 5–26 (2005).
5) Chapple CR, Khullar V, Gabriel Z, Muston D, Bitoun CE, Weinstein D. The effects of antimuscarinic treatments in overactive bladder: an update of a systematic review and meta-analysis. Eur. Urol., 54, 543–562 (2008).
6) Araújo H, Akishita M, Teramoto S, Araújo H, Mizukami K, Morimoto S, Toka K. Incidence of adverse drug reactions in geriatric units of university hospitals. Geriatr. Gerontol. Int., 5, 293–297 (2005).
7) Callegari E, Malhotra B, Bungay PJ, Webster R, Fenner KS, Kempshall S, LaPerle JL, Michel MC, Kay GG. A comprehensive non-clinical evaluation of the CNS penetration potential of antimuscarinic agents for the treatment of overactive bladder. Br. J. Clin. Pharmacol., 72, 235–246 (2011).
8) Ancelin ML, Artero S, Portel F, Dupuy A-M, Touchon J, Ritchie K. Non-degenerative mild cognitive impairment in elderly people and use of anticholinergic drugs: longitudinal cohort study. BMJ, 332, 453–459 (2006).
9) Kopolov MD. The cholinergic neurotransmitter system in human memory and dementia: a review. Q. J. Exp. Psychol. A, 38, 555–572 (1986).
10) Scheid R, Takeda M. Central nervous system safety of anticholinergic drugs for the treatment of overactive bladder in the elderly. Clin. Ther., 27, 144–153 (2005).
19) Annaert PP, Brouwer KL. Assessment of drug interactions in hepatocytes. *Biol. Pharm. Bull.*, **33**, 1238–1241 (2010).

18) Nare B, Prichard RK, Georges E. Characterization of rhodamine 123 binding to P-glycoprotein and peptide transporters in corneal epithelial cells—novel strategies for intracellular drug targeting. *Exp. Eye Res.*, **106**, 47–54 (2013).

17) Hunter J, Jepson MA, Tsuruo T, Simmons NL, Hirst BH. Functional expression of P-glycoprotein in apical membranes of human intestinal Caco-2 cells. Kinetics of vinblastine secretion and intercellular P-glycoprotein. *Basic Clin. Pharmacol. Toxicol.*, **113**, 259–265 (2013).

16) Elsby R, Surry DD, Smith VN, Gray AJ. Validation and application of Caco-2 assays for the in vitro evaluation of development candidate drugs as substrates or inhibitors of P-glycoprotein to support regulatory submissions. *Int. J. Pharm.*, **388–394** (2005).

15) Zhang X, Qiu F, Jiang J, Gao C, Tan Y. Intestinal absorption mechanisms of berberine, palmatine, jateorhizine, and coptisine: involvement of P-glycoprotein. *Arch. Biochem. Biophys.*, **41**, 290–296 (2011).

14) Barot M, Gokulgandhi MR, Pal D, Mitra AK. Mitochondrial localization of P-glycoprotein and peptide transporters in corneal epithelial cells. *Basic Clin. Pharmacol. Toxicol.*, **113**, 259–265 (2013).

13) Gschwind L, Rollason V, Daali Y, Bonnabry P, Dayer P, Desmeules JA. Role of P-glycoprotein in the uptake/efflux transport of oral vitamin K antagonists and rivaroxaban through the Caco-2 cell model. *Basic Clin. Pharmacol. Toxicol.*, **113**, 259–265 (2013).

12) Wakuda H, Nejime N, Tada Y, Kagota S, Fahmi OA, Umegaki K, Yamada S, Shinozuka K. A novel method using confocal laser scanning microscopy for sensitive measurement of P-glycoprotein-mediated transport activity in Caco-2 cells. *J. Pharmacol. Pharmacokinet.*, **36**, 1015–1021 (2011).

11) Wakuda H, Nejime N, Tada Y, Kagota S, Fahmi OA, Umegaki K, Yamada S, Shinozuka K. Highly sensitive measurement of P-glycoprotein-mediated transport activity in Caco-2 cells. *Biol. Pharm. Bull.*, **33**, 1238–1241 (2010).

10) Kageyama M, Namiki H, Fukushima H, Ito Y, Shibata N, Takada K. In vivo effects of cyclosporine A and ketoconazole on the pharmacokinetics of representative substrates for P-glycoprotein and cytochrome P450 (CYP) 3A in rats. *Biol. Pharm. Bull.*, **28**, 316–322 (2005).

9) Parasrampuria R, Mehvar R. Effects of P-glycoprotein and Mrp2 inhibitors on the hepatobiliary disposition of Rhodamine 123 and its glucuronidated metabolite in isolated perfused rat livers. *J. Pharm. Sci.*, **99**, 455–466 (2010).

8) Jouan E, Le Véc M, Mayati A, Denizot C, Parmentier Y, Fardel O. Evaluation of P-glycoprotein inhibitory potential using a rhodamine 123 accumulation assay. *Drug Metab. Dispos.*, **8**, 12 (2016).

7) Lee JS, Paull K, Alvarez M, Hose C, Monks A, Grever M, Fojo AT, Bates SE. Rhodamine efflux pattern predict P-glycoprotein substrates in the National Cancer Institute drug screen. *Mol. Pharmacol.*, **46**, 627–638 (1994).

6) van der Sandt IC, Blom-Roosemalen MC, de Boer AG, Breimer DD. Specificity of doxorubicin versus rhodamine-123 in assessing P-glycoprotein functionality in the LLC-PK1, LLC-PK1:MDR1 and Caco-2 cell lines. *Eur. J. Pharmacol.*, **11**, 207–214 (2000).

5) Thomas H, Coley HM. Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting P-glycoprotein. *Cancer Control*, **10**, 159–165 (2003).

4) Miller DW, Hinton M, Chen F. Evaluation of drug efflux transporter liabilities of darifenacin in cell culture models of the blood–brain and blood–ocular barriers. *Neurol. Urodyn.*, **30**, 1633–1638 (2011).

3) Skerjanec A. The clinical pharmacokinetics of darifenacin. *Clin. Pharmacokinet.*, **45**, 325–350 (2006).

2) Koren G, Woodland C, Ito S. Toxic digoxin-drug interactions: The case for a sensitive system to assess drug interactions. *Annu. Rev. Pharmacol. Toxicol.*, **46**, 45–46 (1996).

1) Fromm MF, Kim RB, Stein CM, Wilkinson GR, Roden DM. Inhibition of P-glycoprotein-mediated drug transport: a unifying mechanism to explain the interaction between digoxin and quinidine. *Circulation*, **99**, 552–557 (1999).

1) Darifenacin, Enablex®, prescribing information. Novartis Pharmaceuticals Corporation, East Hanover, NJ, U.S.A. (2004).

1) Sandage B, Sabounjian L, Shipley J, Profy A, Lasseter K, Fox L, Harnett M. Predictive power of an in vitro system to assess drug interactions of an antimuscarinic medication: a comparison of in vitro and in vivo drug-drug interaction studies of trospium chloride with digoxin. *J. Clin. Pharmacol.*, **46**, 776–784 (2006).

1) Mukhametov A, Raevsky OA. On the mechanism of substrate/non-substrate recognition by P-glycoprotein. *J. Mol. Graph. Model.*, **7**, 227–232 (2017).

1) Srikrishna S, Robinson D, Cardozo L. Important drug–drug interactions for treatments that target overactive bladder syndrome. *Int. Urogynecol. J.*, **25**, 715–720 (2014).