In Vivo Quantitative Autoradiographic Analysis of Brain Muscarinic Receptor Occupancy by Antimuscarinic Agents for Overactive Bladder Treatment

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ABBREVIATIONS: OAB, overactive bladder; [11C](+)3-MPB, (+)N-[11C]methyl-3-piperidyl benzilate; CNS, central nervous system; REM, rapid eye movement; RO, receptor occupancy; qEEG, quantitative electroencephalography; BBB, blood-brain barrier; QNB, quinuclidinyl benzilate
ABSTRACT

We evaluated the effects of five clinically used antimuscarinic agents for overactive bladder (OAB) treatment on in vivo muscarinic receptor binding in rat brain by quantitative autoradiography. There was a dose-related decrease in in vivo specific (+)N-[^11C]methyl-3-piperidyl benzilate ([^11C](+)3-MPB) binding in each brain region of rats 10 min after i.v. injection of oxybutynin, propiverine, solifenacin and tolerodine. Rank order of the i.v. dose (RO_{50}) for 50% receptor occupancy of antimuscarinic agents in rat brain regions was propiverine > solifenacin > tolerodine, oxybutynin. There was a good linear relationship between in vivo (pRO_{50} values in the rat hippocampus) and in vitro (pK_{i} values in human M_{1} receptors) receptor binding activities of propiverine, solifenacin and tolerodine. The observed RO_{50} value of oxybutynin was about five times smaller than the predicted in vitro K_{i} value. The dose ratios of antimuscarinic agents for the brain receptor occupancy (RO_{50}) to the inhibition of carbachol- and volume-induced increase in intravesical pressure (ID_{50}), which reflects in vivo selectivity for the urinary bladder over the brain, were greater for solifenacin, tolerodine and propiverine than oxybutynin. Darifenacin displayed only slight decrease in specific [^11C](+)3-MPB binding in the rat brain regions and it was not dose-related. In conclusion, in vivo quantitative autoradiographic analysis of brain muscarinic receptor occupancy may provide fundamental basis for managing CNS side effects in antimuscarinic therapy for OAB. It is suggested that in the treatment of OAB, CNS side effects can be avoided by antimuscarinic agents with high selectivity for the urinary bladder over the brain.
**Introduction**

Antimuscarinic agents are widely used as the first line therapy for urgency, frequency with or without urge incontinence, all symptoms of the disorder termed overactive bladder (OAB). Antimuscarinic treatment is frequently associated with side-effects that limit its clinical use (Andersson et al., 2004). Because the muscarinic receptor mediates the excitatory and inhibitory actions of acetylcholine in the central and peripheral nervous systems, various systemic adverse effects may occur by the administration of antimuscarinic agents for OAB. In fact, dry mouth occurs most commonly and thereby decreases the quality of life of patients. Therefore numerous studies involving antimuscarinic agents to treat OAB have focused to the selectivity for the urinary bladder over the salivary gland. The incidence of central nervous system (CNS) side effects by antimuscarinic agents is generally lower than that of dry mouth, but CNS side-effects may be of great concern in elderly patients because of an increase of blood-brain barrier (BBB) permeability with aging (Pakulski et al., 2000; Ouslander, 2004). In fact, short-term and chronic administration of oxybutynin in elderly subjects resulted in a non-degenerative mild cognitive dysfunction (Katz et al., 1998; Ancelin et al., 2006). Moreover, clinical studies have demonstrated the increased cognitive sensitivity to scopolamine (Flicker et al., 1992; Molchan et al., 1992) and a reduced density of brain muscarinic receptors in the elderly (Norbury et al., 2004).

Tolterodine seems to be less associated with cognitive impairment than oxybutynin as it is less lipophilic and, therefore, less likely to cross BBB (Chapple, 2000; Clemett and Jarvis, 2001). However, Diefenbach et al. (2005) showed that oxybutynin and tolterodine administration resulted in statistically significant reductions in rapid eye movement (REM) sleep and slight increase in REM latency. Newly developed antimuscarinic agents such as solifenacin and darifenacin compared with oxybutynin may exert relatively fewer CNS effects...
than oxybutynin. Darifenacin did not affect cognitive function in elderly subjects (Lipton et al., 2005; Kay et al., 2005, 2006). Solifenacin compared with oxybutynin has shown less effect on the acquisition and consolidation of memory by passive avoidance test in rats (Suzuki et al., 2007). To our knowledge, the CNS effect of propiverine was little reported previously.

Antimuscarinic agents are considered to exert CNS side effects by binding to brain muscarinic receptors. Oki et al. (2007) have recently shown that oral administration of oxybutynin but not tolterodine and darifenacin bound significantly to muscarinic receptors in the mouse brain. However, these authors examined the effects of only single and low dose of each agent on brain muscarinic receptor binding. Oxybutynin, propiverine and tolterodine form active metabolites after oral administration and thus, these metabolites may contribute to the efficacy and tolerability in vivo (Michel and Hegde, 2006). Because metabolites are generally more hydrophilic than the parent agents, however, the CNS pharmacological effect may be mainly due to the parent agents. In fact, the brain concentration of active metabolite (desethyloxybutynin) after oral oxybutynin (0.25-2.54 µmol/kg) was less than 1% when compared with that of the parent compound (Maruyama et al., unpublished observation). To clarify the risk of CNS side-effects in OAB treatment by antimuscarinic agents, therefore, we have quantitatively compared brain muscarinic receptor binding in rats after the i.v. injection of oxybutynin, propiverine, solifenacin, tolterodine and darifenacin (Fig. 1A) at four different doses.
Materials and Methods

Animals. Male Sprague-Dawley rats (270 - 320 g) at the age of 8 weeks were purchased from Shizuoka Laboratory Co. Ltd. (Shizuoka, Japan). They were housed in the laboratory with free access to food and water, and maintained on a 12-hr dark/light cycle in a room with controlled temperature (24 ± 1°C) and humidity (55 ± 5%). This study was conducted in accordance with the guide for care and use of laboratory animals as adopted by the US National Institutes of Health.

Materials. (+)N-[11C]methyl-3-piperidyl benzilate ([11C](+)-3-MPB) (Fig. 1B) was synthesized by N-methylation of nor-compound (+)3-piperidyl benzilate with [11C]methyl iodide (Takahashi et al., 1999; Tsukada et al., 2001). Oxybutynin hydrochloride was purchased from Sigma Aldrich (St. Louis, MO, USA). Propiverine hydrochloride, solifenacin succinate, tolterodine L-tartrate and darifenacin hydrobromide were donated from Taiho Pharmaceutical Co., Ltd. (Tokushima, Japan), Astellas Pharma Inc. (Tokyo, Japan), Pharmacia Co. Ltd. (Tokyo, Japan) and Pfizer Co. Ltd. (Tokyo, Japan), respectively. All other chemicals were purchased from commercial sources. Darifenacin was dissolved in dimethylsulfoxide and diluted in physiological saline. [11C][(+)-3-MPB and the other compound were dissolved and diluted in physiological saline. The injection volumes of [11C][(+)-3-MPB and antimuscarinic agents were 0.5 and 0.25 mL, respectively.

Animal treatment and autoradiography. Rats intraperitoneally anesthetized with chloral hydrate (400 mg/kg) received an i.v. injection of saline, oxybutynin (0.08 - 2.54 µmol/kg), propiverine (0.74 - 24.8 µmol/kg), solifenacin (0.62 - 20.8 µmol/kg), tolterodine (0.21 - 6.31 µmol/kg) or darifenacin (0.20 - 5.91 µmol/kg). At 10 min after the injection, [11C](+)-3-MPB
(150 MBq) was intravenously injected. At 30 min after the injection of \[^{11}C\](+)3-MPB, rats were sacrificed and the brain tissues were rapidly removed for the autoradiography. The brains were cut into 2 mm thick coronal sections using a brain matrix (Muromachi Co. Ltd., Kyoto, Japan) at room temperature. The sections were placed on an imaging plate for 10 min, and the plate was scanned with a bio-imaging analyzer of type BAS 1500 (FUJIX Fuji Photo Film Co.Ltd., Tokyo, Japan). Regions of interest were placed on the cerebral cortex, corpus striatum, hippocampus, amygdala, thalamus, hypothalamus, pons and cerebellum by using a Macintosh computer (Image Reader version 1.2, Fuji film). The value of pmol/g wet tissue was calculated from the value of photo-stimulated luminescence unit/mm\(^2\). For the quantification, individual calibration standards were prepared for each set of brain sections exposed to the same imaging plate. The standard (10 \(\mu\)l) of \[^{11}C\](+)3-MPB solution at the known concentration was placed on an Advantec filter paper No.2 (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and exposed simultaneously with the brain sections. From the radioactivity of standard sample, the amount of substance expressed in Bq was calculated. The total counts over the standard, measured by the phosphor-imaging system, allowed the calculation of a calibration factor in counts per Bq. The signal measured for region of interest in a structure of a brain slice was given as average counts per pixel. From the pixel size, the value to counts/mm\(^2\) was calculated and, using the calibration factor, it was converted to MBq/mm\(^2\) (and MBq/g tissue). Before the calculation, the average counts per pixel of the background area close to the brain slices were subtracted from the average counts of the region of interest. The value of pmol/g tissue was estimated from specific activity of \[^{11}C\](+)3-MPB. The data were corrected for decay.

Specific binding of \[^{11}C\](+)3-MPB was defined as the difference of radioactivity between each brain region and cerebellum because even high dose of atropine failed to lower \textit{in vivo} \[^{3}H\]quinuclidinyl benzilate (QNB) binding in the rat cerebellum (Yamamura et al., 1974). Receptor occupancy (RO) was calculated from the degree of reduction (%) by antimuscarinic
agents in *in vivo* specific $[^{11}C](+)$3-MPB binding in each brain region.

**Data analysis.** The dose-receptor occupancy curves were fitted to following hyperbolic function with an assumption of single binding site by GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). RO values were calculated by the following equation (1):

$$
\text{RO} (%) = \frac{[D]^\gamma}{[D]^\gamma + [\text{RO}_{50}]^\gamma} \times 100 \quad \text{..................(1)}
$$

where RO is the receptor occupancy (%), D is the dose (µmol/kg) and $\gamma$ is the Hill coefficient. $\text{RO}_{50}$ is the dose at which a half-maximal receptor occupancy is obtained. The graphs shown in the figures were drawn using the fitting constants.

Because the $M_1$ subtype is the most abundant in hippocampus which plays important roles in working and reference memory, the binding affinities (inhibition constant: $K_i$) of antimuscarinic agents in human recombinant $M_1$ receptor subtypes were used as an index of *in vitro* affinities of these agents (Maruyama et al., 2006). A relationship between *in vivo* ($\text{RO}_{50}$ values in rat hippocampus) and *in vitro* ($K_i$ values in human $M_1$ receptor) affinities of antimuscarinic agents were examined by a linear regression analysis using GraphPad Prism 4 (GraphPad Software Inc., San Diego CA). The ratio for brain receptor occupancy ($\text{RO}_{50}$) to the pharmacological potency in the urinary bladder ($\text{ID}_{50}$) of antimuscarinic agents was calculated. The $\text{ID}_{50}$ value, the dosage of antimuscarinic agent needed to inhibit 50% of carbachol- and volume-induced increases in the intraurethral pressure in rats, was cited from previous literatures (Ikeda et al., 2002; Modiri et al., 2002; Ohtake et al., 2004).

All data are expressed as the mean ± SEM or with 95% confidence interval (CI). The statistically significant difference was analyzed by Williams’s test. Difference with $p<0.05$ were considered statistically significant.
Results

Fig. 2A illustrates typical autoradiographic images in the rat brain at 30 min after the i.v. injection of \([^{11}\text{C}](+)^{3}\text{-MPB}\). The highest radioactivity of \([^{11}\text{C}](+)^{3}\text{-MPB}\) was detected in the corpus striatum and the lowest value was seen in the cerebellum. This agrees well with the previous finding by Yamamura and Snyder (1974) who showed the rank of regional distribution of muscarinic receptors in the rat brain, being corpus striatum > cerebral cortex > hippocampus > midbrain and pons, thalamus, hypothalamus >> cerebellar cortex. Even high dose of atropine failed to lower \textit{in vivo} \([^{3}\text{H}]\text{QNB}\) binding in the rat cerebellum (Yamamura et al., 1974).

The effects of i.v. injection of antimuscarinic agents were examined on specific \([^{11}\text{C}](+)^{3}\text{-MPB}\) binding in the cerebral cortex, corpus striatum, hippocampus, amygdala, thalamus, hypothalamus and pons of rats. As shown in Fig. 2B, there was a dose-dependent decrease in the radioactivity of \([^{11}\text{C}](+)^{3}\text{-MPB}\) in the brain of rats 10 min after the i.v. injection of oxybutynin (0.08 - 2.54 µmol/kg), propiverine (0.74 - 24.8 µmol/kg), solifenacin (0.62 - 20.8 µmol/kg) and tolterodine (0.21 - 6.31 µmol/kg). In the case of darifenacin (0.20 - 5.91 µmol/kg), slight decrease in the radioactivity was also observed, but it was not the dose-related. From the data in Fig. 2B, we estimated \textit{in vivo} specific \([^{11}\text{C}](+)^{3}\text{-MPB}\) binding in each brain region after the i.v. injection of antimuscarinic agents (Fig. 3). Following the i.v. injection of oxybutynin (0.08 - 2.54 µmol/kg), specific \([^{11}\text{C}](+)^{3}\text{-MPB}\) binding was significantly decreased in the hippocampus (20.3 - 74.3%), amygdala (33.8 - 76.5%), thalamus (34.1 - 73.0%) and hypothalamus (33.9 - 75.4%) in a dose-dependent manner. The i.v. injection of oxybutynin (0.25 - 2.54 µmol/kg) showed a significant decrease of specific \([^{11}\text{C}](+)^{3}\text{-MPB}\) in the cerebral cortex (41.4 - 75.8 %), corpus striatum (35.8 - 74.7%) and pons (41.9 - 73.2%).

Similarly, i.v. injection of propiverine (7.43, 24.8 µmol/kg) showed significant and
dose-dependent decreases in specific $[^{11}\text{C}]$(+3-MPB binding in the cerebral cortex (33.5%, 56.8%), hippocampus (26.7%, 49.6%), amygdala (34.2%, 54.6%), thalamus (40.5%, 65.2%), hypothalamus (44.0%, 70.6%) and pons (39.7%, 59.7%). In the corpus striatum, significant decreases of specific $[^{11}\text{C}]$(+3-MPB binding by 24.8 µmol/kg propiverine was 40.2%. This agent at 0.74 and 2.48 µmol/kg had little significant effect on the brain $[^{11}\text{C}]$ (+3-MPB binding.

Solifenacin at i.v. doses of 2.08 to 20.8 µmol/kg induced significant decreases in specific $[^{11}\text{C}]$(+3-MPB binding in all brain regions compared with control values, and the decreases in the cerebral cortex, corpus striatum, hippocampus, amygdala, thalamus, hypothalamus and pons ranged from 30.6% to 78.3%. This effect at these doses seemed to be not necessarily the dose-related. The lower dose (0.62 µmol/kg) of solifenacin had little significant effect on the brain $[^{11}\text{C}]$(+3-MPB binding.

Following i.v. injection of tolterodine (0.21 - 6.31 µmol/kg), specific $[^{11}\text{C}]$(+3-MPB binding was significantly decreased in the hippocampus (21.0 - 90.2%), amygdala (25.7% - 91.6%), thalamus (31.3 - 90.5%), hypothalamus (35.1 - 95.6%) and pons (43.8 - 90.6%) in a dose-dependent manner. The i.v. injection of tolterodine (0.63 - 6.31 µmol/kg) showed a significant decrease of specific $[^{11}\text{C}]$(+3-MPB in the cerebral cortex (48.2 - 91.4%) and corpus striatum (43.5 - 91.4%). In contrast, darifenacin (0.20 - 5.91 µmol/kg) displayed little significant change in specific $[^{11}\text{C}]$ (+3-MPB binding.

On the basis of the data in Fig. 3, we estimated the dose-receptor occupancy curves in each brain region after i.v. injection of oxybutynin, proiverine, solifenacin and tolterodine (Fig. 4, Table 1). As shown in Table 1, the rank order of average RO$_{50}$ values of antimuscarinic agents in the rat brain regions was propiverine (10.0 - 40.5 µmol/kg) > solifenacin (3.3 - 10.7 µmol/kg) > tolterodine (0.34 - 0.98 µmol/kg), oxybutynin (0.28 - 0.57 µmol/kg). In the case of darifenacin, the RO$_{50}$ value could not be estimated by the lack of dose-dependent inhibition of
specific $[^{11}C](+)^3$-MPB binding. The average RO$_{50}$ values of oxybutynin seemed to be similar among each brain region, while RO$_{50}$ values of propiverine and tolterodine tended to be larger in the corpus striatum and hippocampus than in the hypothalamus andpons.

Fig. 5 shows a relationship between in vivo (pRO$_{50}$ values in the rat hippocampus) and in vitro (p$K_i$ values in human M$_1$ receptors, Maruyama et al., 2006) receptor binding affinity of antimuscarinic agents. The linear regression analysis between propiverine, solifenacin and tolterodine showed a good fitness ($r^2 = 0.9999$) by the equation of pRO$_{50} = 0.6946 \times pK_i - 0.001054$. By this equation, the in vivo affinity (pRO$_{50}$ value) of these agents from in vitro affinity (p$K_i$ value) could be predicted. Table 2 compares the observed and predicted values of RO$_{50}$ in the rat hippocampus of antimuscarinic agents with their ratios after the i.v injection. The predicted values of propiverine, solifenacin and tolterodine corresponded nicely to the observed values, but the predicted value of oxybutynin was about five times larger than the observed value.

The selectivity for the urinary bladder over the brain region of antimuscarinic agents in rats was evaluated by ratios for the brain receptor occupancy (RO$_{50}$) to the pharmacological potency in the urinary bladder (ID$_{50}$). The pharmacological potency (ID$_{50}$) of each agent in the urinary bladder was cited from previous literatures of inhibitory effects of carbachol- and volume-induced increases in the intravesical pressure of rats (Ikeda et al., 2002; Modiri et al., 2002; Ohtake et al., 2004). The rank order of the ratio (RO$_{50}$/ID$_{50}$) in brain regions to the urinary bladder was solifenacin (8.1 - 46.7), tolterodine (3.6 - 17.9), propiverine (2.2 - 8.9) > oxybutynin (1.4 - 3.4) (Table 3). Thus, the selectivity for the urinary bladder over the hippocampus was greater for solifenacin (11.2, 20.0), tolterodine (9.5, 16.3) and propiverine (5.4) than oxybutynin (2.5 - 3.0). Similar tendency was observed also in other brain regions.
Discussion

The brain muscarinic receptor was measured by quantitative autoradiographic analysis in rats after i.v. injection of \([^{11}\text{C}](+3\text{-MPB}).\) \([^{11}\text{C}](+3\text{-MPB}\) may be a superior radioligand for the \textit{in vivo} labeling of brain muscarinic receptors because of lower binding affinity and rapid equilibrium in comparison with other radioligands such as \([^{3}\text{H}]\)QNB (Takahashi et al., 1999; Tsukada et al., 2001). The autoradiographic technique to measure various receptors is well established with long-lived radiotracers (e.g. \(^3\text{H}\) and \(^{14}\text{C}\) etc), but the major disadvantage is to take several weeks as the exposure time of film for the data analysis due to the low-energy emitting radionuclides. In contrast, the merit of use of high-energy \(\beta^+\)- emitting radionuclides such as \(^{11}\text{C}\) or \(^{18}\text{F}\) is to make the exposure time markedly shorter, being usually only several minutes or hours. Moreover, the autoradiographic method using \(\beta^+\)- emitting radionuclides could apply to positron emission tomography. The \textit{in vivo} competition binding assay by the quantitative autoradiographic method is a powerful way to analyze directly muscarinic receptor occupancy of antimuscarinic agents in the brain, making possible to quantify the penetration of antimuscarinic agents into brain tissues.

To clarify the risk of CNS side-effects in OAB treatment, using the quantitative autoradiographic method, we have comparatively characterized brain muscarinic receptor binding after the i.v. injection of oxybutynin, propiverine, solifenacin, tolterodine and darifenacin. The i.v. doses of antimuscarinic agents used in the present study were determined on the basis of pharmacologically relevant doses to impair learning in rat passive avoidance response (Suzuki et al., 2007). The i.v. injection of oxybutynin, propiverine, tolterodine and solifenacin significantly decreased \textit{in vivo} specific \([^{11}\text{C}](+3\text{-MPB}\) binding in each brain region of rats in a dose-dependent manner. According to the estimated RO\(_{50}\) values, the potency of muscarinic receptor occupancy by each agent in the rat brain appeared to be greatest in oxybutynin,
followed by tolterodine, solifenacin and proiverine. Further, it was shown that \textit{in vivo} potencies (pRO$_{50}$) of propiverine, solifenacin and tolterodine in occupying \textit{in vivo} hippocampal receptors after the i.v. injection correlated well with their \textit{in vitro} binding affinities in human M$_1$ muscarinic receptor subtype (Maruyama et al., 2006) (Fig. 5). However, the observed RO$_{50}$ value of oxybutynin was about five times smaller than the value predicted by the linear correlation between propiverine, solifenacin and tolterodine. These data suggest that oxybutynin crosses more easily through BBB than other antimuscarinic agents examined.

Antimuscarinic agents must first pass the BBB to occupy central muscarinic receptors. The observed difference among antimuscarinic agents in the potency of brain muscarinic receptor occupancy may be defined by BBB permeability which is responsible for CNS effects in patients. The passive penetration of antimuscarinic agents through this physiologic barrier generally depends on physicochemical factors such as high lipophilicity, low degree of ionization (neutral charge) and small molecular size (Sheife and Takeda, 2005). The characteristics of chemical properties of oxybutynin relative to tolterodine - lipophilicity (Log $K_{ow}$: 4.68 vs 1.83) (Watanabe, 2007; Abrams, 2001) and neutral polarity (pKa: 6.44 vs 9.87) (Yokoyama et al., 1996; Abrams, 2001) - make it the most likely to cross the BBB (Kay and Granville, 2005). Påhlman et al. (2001) have revealed that the distribution of radioactivity in the brain was the lowest among other tissues from the mice received oral $[^{14}C]$tolterodine. This may be consistent with the relatively lower \textit{in vivo} binding activity of brain muscarinic receptors by tolterodine compared with the pharmacological activity in the bladder. Also, solifenacin, like tolterodine, may have molecular characteristics that make it unlikely to cross the BBB.

RO$_{50}$ values of antimuscarinic agents (Table 1) were similar to i.v. doses inhibiting learning/memory behavior in rats (Suzuki et al., 2007). Thus, these values could be regarded as the index of CNS pharmacological effects following the blockade of brain muscarinic
receptors. Subsequently, we evaluated the selectivity for the urinary bladder over the brain of antimuscarinic agents by using pharmacological data in the rat urinary bladder (Ohtake et al., 2004; Ikeda et al., 2002; Modiri et al., 2002). It was shown that antimuscarinic agents inhibited significantly carbachol- and volume-induced increase in the intravesical pressure in rats. The dose ratios (RO<sub>50</sub>/ID<sub>50</sub>) of antimuscarinic agents for the brain receptor occupancy to the inhibitory potency of increases in intravesical pressure were considered to reflect in vivo pharmacological selectivity for the urinary bladder over the brain. This ratio was relatively large in solifenacin (8.1 - 46.7), tolterodine (3.6 - 17.9) and propiverine (2.2 - 8.9), compared with oxybutynin (1.4 - 3.4) (Table 3). Thus, the selectivity for the urinary bladder over the brain was relatively low for oxybutynin, suggesting a high feasibility of CNS side effects at pharmacological doses to treat OAB. This finding seems to be in a reasonable agreement with previous observations of oxybutynin shown by pharmacological (Sugiyama et al., 1999) and ex vivo receptor binding (Oki et al., 2001) studies in rats, and also by clinical studies (Katz et al., 1998; Pietzko et al., 1994; Todorova et al., 2001; Ancelin et al., 2006). In fact, CNS side effect by oxybutynin occurred in patients with OAB and in older subjects receiving this drug (Scheife and Takeda, 2005; Kay et al., 2006). It was shown that only higher dose (24.8 µmol/kg i.v.) of propiverine induced CNS effects in rats (Suzuki et al., 2007) and bound to muscarinic receptors in rat brain (Oki et al., 2001). Thus, propiverine may exhibit lower incidence of CNS effects than that of oxybutynin in patients with OAB.

The selectivity for the urinary bladder over the brain of solifenacin and tolterodine was clearly higher than that of oxybutynin in rats. In clinical study, the incidence of CNS side effect of tolterodine was shown to be lower than that of oxybutynin and comparable to that with placebo (Chapple, 2000; Clemett and Jarvis, 2001; Scheife and Takeda, 2005). Thus, these data suggest that solifenacin and tolterodine have advantages in the treatment of OAB owing to lower CNS effect. Todorova et al. (2001) comparatively evaluated electrophysiologic
effects of antimuscarinic agents on the CNS in healthy male volunteers by using quantitative electroencephalography (qEEG). They found that oxybutynin significantly altered qEEG activity, while tolterodine and trospium induce only a slight effect on qEEG activity. Similar electrophysiological results in healthy males with oxybutynin have been shown by Pietzko et al. (1994).

Darifenacin at pharmacologically effective doses did not significantly reduce in vivo specific [11C](+) 3-MPB binding so that the RO50 value could not be estimated. These data suggest that darifenacin induces the lowest incidence of CNS effects among antimuscarinic agents examined. This result agrees with our previous ex vivo findings (Oki et al., 2007) and previous clinical observations (Lipton et al., 2005; Kay et al., 2005, 2006), showing that the treatment with darifenacin may have little effect on the cognitive function in the elderly subjects. Several lines of evidence suggest that darifenacin is unlikely to cross the BBB. Darifenacin is a substrate for P-glycoprotein (Skerjanec, 2006), an active-transporter system that carry back across the BBB.

Additionally, muscarinic receptor subtype selectivity of antimuscarinic agents may be implicated in the appearance of CNS effect. All of five muscarinic receptor subtypes are expressed in the brain (Flynn et al., 1997; Oki et al., 2005). The M1 receptor was abundant in the cortex and hippocampus. In the striatum, the M1 and M4 receptors were distributed. By contrast, the M2 receptor was predominantly localized in the brainstem and cerebellum. The M3 receptor displays lower density in the brain when compared to M1, M2 and M4 receptor expressions. The cognitive dysfunction by antimuscarinic agents may be mediated mainly by the M1 and M2 receptors in the CNS (Kay et al., 2005). Oxybutynin shows selectivity for the M1, M3 and M4 receptors, while tolterodine and propiverine are relatively nonselective to muscarinic receptor subtypes (Maruyama et al., 2006; Ohtake et al., 2007). Solifenacin and darifenacin show higher selectivity to the M3 receptor than M1, M2 and M4 receptors. Thus, M1
selectivity in addition to high BBB permeability of oxybutynin may be more apt to cause CNS side effects. Such muscarinic receptor subtype selectivity may be partly associated with an observed difference among antimuscarinic agents in the in vivo potency of brain receptor occupancy (Fig. 4, Table 1). Taken together, the present study has basically confirmed ex vivo binding data in mouse brain obtained by single oral dose of oxybutynin, tolterodine and darifenacin (Oki et al., 2007), and greatly expanded their findings by using four different i.v. doses of clinically used five antimuscarinic agents under in vivo condition.

In conclusion, analysis of brain muscarinic receptor occupancy in vivo may provide fundamental basis for antimuscarinic agent-induced CNS side effects in OAB treatment. The use in OAB patients would require careful dose adjustment to avoid the risk of side effects associated with brain receptor binding. Relatively newer generation of antimuscarinic agents which have high selectivity of the urinary bladder rather than the brain could be advantageous to treat OAB.
References


Footnotes

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LEGENDS FOR FIGURES

Fig. 1. Chemical structures of oxybutynin, propiverine, tolterodine, solifenacin and darifenacin (A), and [11C](+)-3-MPB (B).

Fig. 2. (A) Representative autoradiographic images (distribution of radioactivity) in the brain regions of rats received i.v. injection of [11C](+)-3-MPB. At 30 min after i.v. injection of [11C](+)-3-MPB (150 MBq) in rats, brain tissues were removed and cut every 2 mm as a center of thalamus and analyzed for the autoradiography. (B) Effects of i.v. injection of different doses of oxybutynin, propiverine, solifenacin, tolterodine and darifenacin on autoradiographic images of [11C](+)-3-MPB in rat brain. Rats received i.v. injection of different doses of each antimuscarinic agent, and at 10 min later, they received i.v. injection of [11C](+)-3-MPB (150 MBq). At 30 min later, brain tissues were removed.

Fig. 3. Effects of i.v. injection of oxybutynin, propiverine, solifenacin, tolterodine and darifenacin on in vivo specific [11C](+)-3-MPB binding in rat brain regions (cerebral cortex, corpus striatum, hippocampus, amygdala, thalamus, hypothalamus and pons). Rats received i.v. injection of [11C](+)-3-MPB (150 MBq) 10 min after i.v. injection of oxybutynin, propiverine, solifenacin, tolterodine and darifenacin. Specific [11C](+)-3-MPB binding was determined as described in Materials and Methods. Each column represents the mean ± SEM of three rats. *, ** Significantly different from the control values (vehicle), *p<0.05, **p<0.01.

Fig. 4. Dose-muscarinic receptor occupancy curves in each brain region of rats after i.v. injection of oxybutynin (●), propiverine (▲), solifenacin (○) and tolterodine (△). Rats received i.v. injection of [11C](+)-3-MPB (150 MBq) 10 min after i.v. injection of oxybutynin, propiverine, solifenacin and tolterodine. The muscarinic receptor occupancy was determined from the degree of reduction (%) by antimuscarinic agents of in vivo specific [11C](+)-3-MPB.
binding in each brain region as described in Materials and Methods. Each point represents the mean ± SEM of three rats.

**Fig. 5.** Relationship between *in vivo* hippocampal receptor occupancy (average pRO_{50}) after i.v. injection of oxybutynin, propiverine, solifenacin and tolerodine (Table 1) and their *in vitro* binding affinities (pK_{i} values) of human recombinant M_{1} receptors. The *in vitro* binding affinities data (pK_{i} values) of human recombinant M_{1} receptors were obtained from Maruyama et al. (2006). The solid line represents a linear regression of propiverine, solifenacin and tolerodine. The multiple correlation coefficient was 0.9999 and the linear equation was pRO_{50} = 0.6946 \times pK_{i} - 0.001054. The broken lines represent 95% confidence limits of the line. The data represent the mean and 95% confidence interval.
TABLE 1
Brain muscarinic receptor occupancy (RO50 values) of antimuscarinic agents estimated from in vivo competitive inhibition of specific [11C](+)3-MPB binding in rat brain regions

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>Average RO50 values (µmol/kg, i.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxybutynin (95% CI)</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>0.43 (0.31 - 0.60)</td>
</tr>
<tr>
<td>Corpus striatum</td>
<td>0.54 (0.38 - 0.77)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.50 (0.35 - 0.73)</td>
</tr>
<tr>
<td>Amygdala</td>
<td>0.39 (0.23 - 0.66)</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.34 (0.21 - 0.55)</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.28 (0.18 - 0.44)</td>
</tr>
<tr>
<td>Pons</td>
<td>0.57 (0.34 - 0.95)</td>
</tr>
</tbody>
</table>

Rats received i.v. injection of [11C](+)3-MPB (150 MBq) 10 min after i.v. injection of oxybutynin (0.08 - 2.54 µmol/kg), propiverine (0.74 - 24.8 µmol/kg), solifenacin (0.62 - 20.8 µmol/kg), tolterodine (0.21 - 6.31 µmol/kg) and darifenacin (0.20 - 5.91 µmol/kg). Specific [11C](+)3-MPB binding was determined and 50% muscarinic receptor occupancy (RO50) was estimated as described in Materials and Methods.
TABLE 2

Comparison of observed and predicted (from Fig. 5) $\text{RO}_{50}$ values of 50% muscarinic receptor occupancy in the rat hippocampus

<table>
<thead>
<tr>
<th>RO$_{50}$ values (µmol/kg, i.v.)</th>
<th>Oxybutynin</th>
<th>Propiverine</th>
<th>Solifenacin</th>
<th>Tolterodine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed values</td>
<td>0.50</td>
<td>24.7</td>
<td>4.6</td>
<td>0.89</td>
</tr>
<tr>
<td>Predicted values</td>
<td>2.5</td>
<td>24.4</td>
<td>4.7</td>
<td>0.88</td>
</tr>
<tr>
<td>Ratios (Predicted/Observed)</td>
<td>5.0</td>
<td>0.99</td>
<td>1.0</td>
<td>0.99</td>
</tr>
</tbody>
</table>
# TABLE 3
The selectivity of antimuscarinic agents for the urinary bladder over the brain

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>Oxybutynin&lt;sup&gt;a,b,c&lt;/sup&gt;</th>
<th>Propiverine&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Solifenacin&lt;sup&gt;a,c&lt;/sup&gt;</th>
<th>Tolterodine&lt;sup&gt;b,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>2.1 - 2.5</td>
<td>3.8</td>
<td>14.4, 25.8</td>
<td>8.8, 15.1</td>
</tr>
<tr>
<td>Corpus striatum</td>
<td>2.7 - 3.2</td>
<td>8.9</td>
<td>17.0, 30.4</td>
<td>10.4, 17.9</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.5 - 3.0</td>
<td>5.4</td>
<td>11.2, 20.0</td>
<td>9.5, 16.3</td>
</tr>
<tr>
<td>Amygdala</td>
<td>1.9 - 2.3</td>
<td>4.0</td>
<td>8.1, 14.6</td>
<td>6.5, 11.1</td>
</tr>
<tr>
<td>Thalamus</td>
<td>1.7 - 2.0</td>
<td>2.7</td>
<td>19.9, 35.7</td>
<td>6.6, 11.3</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>1.4 - 1.7</td>
<td>2.2</td>
<td>15.0, 26.9</td>
<td>4.3, 7.3</td>
</tr>
<tr>
<td>Pons</td>
<td>2.9 - 3.4</td>
<td>3.2</td>
<td>26.1, 46.7</td>
<td>3.6, 6.2</td>
</tr>
</tbody>
</table>

<sup>RO<sub>50</sub></sup> values for antimuscarinic agents were derived from Table 1.

<sup>a,b,c</sup> Pharmacological potency (<i>ID<sub>50</sub></i>) of each agent in the urinary bladder was derived from inhibitory effects of carbachol- and volume-induced increases in the inravesical pressure in rats by <sup>a</sup>Ikeda et al. (2002), <sup>b</sup>Modiri et al. (2002) and <sup>c</sup>Ohtake et al. (2004)
(A) Autoradiographic images

(B) Oxybutynin
0.08 μmol/kg  0.25 μmol/kg  0.76 μmol/kg  2.54 μmol/kg

Propiverine
0.74 μmol/kg  2.48 μmol/kg  7.43 μmol/kg  24.8 μmol/kg

Solifenacin
0.62 μmol/kg  2.08 μmol/kg  6.24 μmol/kg  20.8 μmol/kg

Tolterodine
0.21 μmol/kg  0.63 μmol/kg  2.10 μmol/kg  6.31 μmol/kg

Darifenacin
0.20 μmol/kg  0.59 μmol/kg  1.97 μmol/kg  5.91 μmol/kg

Linear pseudocoloring of phosphor imager signal units (% of maximum)