**α1-Adrenoceptors and muscarinic receptors in voiding function – binding characteristics of therapeutic agents in relation to the pharmacokinetics**

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In vivo and ex vivo binding of α1-adrenoceptor and muscarinic receptors involved in voiding function is reviewed with therapeutic agents (α1-adrenoceptor antagonists: prazosin, tamsulosin and silodosin; and muscarinic receptor antagonists: oxybutynin, tolterodine, solifenacin, propiverine, imidafenac and darifenacin) in lower urinary tract symptoms. This approach allows estimation of the inhibition of a well-characterized selective (standard) radioligand by unlabelled potential drugs or direct measurement of the distribution and receptor binding of a standard radioligand or radiolabelled form of a novel drug. In fact, these studies could be conducted in various tissues from animals pretreated with radioligands and/or unlabelled novel drugs, by conventional radioligand binding assay, radioactivity measurement, autoradiography and positron emission tomography. In vivo and ex vivo receptor binding with α1-adrenoceptor antagonists and muscarinic receptor antagonists have been proved to be useful in predicting the potency, organ selectivity and duration of action of drugs in relation to their pharmacokinetics. Such evaluations of drug–receptor binding reveal that adverse effects could be avoided by the use of new α1-adrenoceptor antagonists and muscarinic receptor antagonists for the treatment of lower urinary tract symptoms. Thus, the comparative analysis of α1-adrenoceptor and muscarinic receptor binding characteristics in the lower urinary tract and other tissues after systemic administration of therapeutic agents allows the rationale for their pharmacological characteristics from the integrated viewpoint of pharmacokinetics and pharmacodynamics. The current review emphasizes the usefulness of in vivo and ex vivo receptor binding in the discovery and development of novel drugs for the treatment of not only urinary dysfunction but also other disorders.

**Introduction**

Lower urinary tract symptoms, characterized by an increased frequency of micturition, urgency, urge incontinence and urinary obstruction, are very common in the geriatric population, a group that is rapidly increasing in number [1–4]. α1-Adrenoceptor antagonists and muscarinic receptor antagonists are widely used as first-line therapy for lower urinary tract symptoms because of benign prostatic hyperplasia (BPH) and overactive bladder (OAB) [5–8]. Prazosin produces some side effects, such as orthostatic hypotension and dizziness, which are ascribed mainly to a reduction in peripheral resistance mediated through a blockade of vascular α1-adrenoceptors. Tamsulosin and silodosin antagonize α1-adrenoceptor-mediated contractile responses to a greater extent in prostate than in vascular tissues [9–12]. Such selectivity of α1-adrenoceptor-mediated responses in the lower urinary tract was not seen with prazosin. α1-Adrenoceptors are classified into several subtypes [13–15]. The prostate contains predominantly α1A- and α1L-adrenoceptors, and the α1L-adrenoceptor seems to be a variant of the α1A-adrenoceptor subtype [15]. In vitro radioreceptor binding and pharmacological studies demonstrate that prazosin and terazosin are nonselective antagonists of α1-adrenoceptor subtypes, while tamsulosin and silodosin...
are relatively selective antagonists of the $\alpha_{1A}$-adrenoceptor subtype.

While muscarinic receptor antagonists have proved effective in patients with OAB, they are also associated with anticholinergic side effects, including dry mouth, constipation, somnolence and blurred vision, because the muscarinic receptor mediates the excitatory and inhibitory actions of acetylcholine in the central and peripheral nervous systems [7]. Dry mouth is the most common of these complaints and decreases quality of life. Therefore, numerous studies involving muscarinic receptor antagonists to treat OAB have focused on targeting the urinary bladder rather than the salivary gland. The incidence of side effects of muscarinic receptor antagonists on the central nervous system (CNS) is generally lower than that of dry mouth, but such effects may be of great concern in elderly patients because of an increase in blood–brain barrier (BBB) permeability with ageing [8, 16]. In this connection, clinical studies demonstrate increased cognitive sensitivity to scopolamine [17, 18] and a reduced density of brain muscarinic receptors in the elderly [19]. Therefore, it is important to evaluate the bladder selectivity of muscarinic receptor antagonists used to treat OAB for optimal medication.

This review article focuses on ex vivo and in vivo receptor binding of $\alpha_{1A}$-adrenoceptor antagonists and muscarinic receptor antagonists in the lower urinary tract and CNS, and discusses the rationale of its usefulness for the pharmacological characterization of therapeutic agents used for lower urinary tract symptoms.

**Characterization of in vivo drug–receptor binding**

Currently, a number of novel drugs exhibiting target organ specificity, receptor subtype selectivity and long duration of action have been developed to reduce side effects and improve patient compliance. $\alpha_{1A}$-Adrenoceptors and muscarinic receptors in the lower urinary tract and the receptor binding of therapeutically used agents have been extensively characterized by in vitro assay techniques, such as radioreceptor binding assays and the measurement of mRNA and protein expression, in tissue membrane preparations and in recombinant cells expressing certain receptor subtypes [20–22]. However, even if these agents display high affinity for a certain receptor subtype in the target organ in vitro, such receptor binding characteristics may not necessarily assure pharmacological specificity in vivo because various pharmacokinetic and pharmacodynamic factors are not taken into account [23] (Figure 1).

Pharmacokinetics includes the absorption, distribution, excretion and metabolism of drugs, while pharmacodynamics includes the affinity of drugs for receptors, signal transduction and homeostatic mechanisms [23]. The drug–receptor interaction results in a measured effect, and the magnitude of the interaction depends on the concentration of the drug in the biophase and further on its affinity for the receptors. The biophase concentration depends not only on the amount of drugs given, but also on pharmacokinetic factors. Inasmuch as in vivo

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**Figure 1**
Schematic representation of in vivo drug–receptor binding in relation to pharmacokinetics and pharmacodynamics. This modified figure is reproduced from reference [23] with permission.
pharmacological effects may be complicated by various physiological, pharmacokinetic and pharmacodynamic factors (Figure 2), the *in vivo* analysis of drug–receptor binding allows more practical information for drug therapy to assess comparatively the extent and time course of drug–receptor binding in target tissues and nontarget tissues in physiological conditions. The most frequent approach is to study the extent to which an unlabelled drug inhibits specific binding of a well-characterized selective radioligand. The alternative direct approach is to radiolabel a new potential drug and to trace its uptake, distribution and binding in tissues. In these approaches, the binding characteristics (degree and time course) of the novel drug on the receptors in the target and nontargeted tissues can be assessed simultaneously. The demonstration of a quantitative relationship between drug binding *in vivo* and drug effects in patients is used to validate targets for drug action, to correlate pharmacological and physiological effects and to optimize clinical treatment.

**α<sub>1</sub>-Adrenoceptor binding in the prostate**

The α<sub>1</sub>-adrenoceptor binding of [3H]prazosin, [3H]tamsulosin and [3H]silodosin after an intravenous injection is widely distributed in various tissues, including the prostate, of rats [24–28]. There was a close correlation in intravenous dose ranges between the *in vivo* α<sub>1</sub>-adrenoceptor binding activities of these agents in the rat prostate and their functional activities in the lower urinary tract [12, 27–29], indicating that [3H]prazosin, [3H]tamsulosin and [3H]silodosin label pharmacologically relevant α<sub>1</sub>-adrenoceptors in the prostate in physiological conditions. In fact, [3H]tamsulosin may be a suitable radioligand for the *in vivo* labelling of α<sub>1</sub>-adrenoceptors in various tissues, because of its extremely low level of nonspecific binding. The parameters for *in vivo* [3H]tamsulosin binding could be estimated easily in rat tissues, and there is a similarity between *in vitro* and *in vivo* binding parameters for this radioligand in rat tissues [27].

In rats, the α<sub>1A</sub>-adrenoceptor subtype exists predominantly in the prostate and submaxillary gland, while the α<sub>1B</sub>-subtype is predominant in the spleen and liver [30–33]. There was a significant difference between [3H]tamsulosin or [3H]silodosin and [3H]prazosin in the degree and time course of *in vivo* α<sub>1</sub>-adrenoceptor binding in rat tissues [25–28]. The α<sub>1</sub>-adrenoceptor binding of [3H]tamsulosin and [3H]silodosin was significantly longer-lasting in the prostate and submaxillary gland than in the spleen, lung and kidney. These results are consistent with the *ex vivo* observation that the oral administration of tamsulosin and silodosin, despite a rapid decline in plasma concentration, causes sustained occupancy of α<sub>1</sub>-adrenoceptors in the rat prostate and submaxillary gland, but not in the spleen.
Silodosin seems to be a highly selective antagonist for the α1A-adrenoceptor subtype in vivo. Notably, the absence of in vivo α1-adrenoceptor binding of [3H]prazosin, [3H]tamsulosin and [3H]silodosin in the cerebral cortex may be as a result of the limited brain distribution because of poor permeability through the BBB [27, 28]. This idea is supported by the ex vivo observation that there is little alteration in α1-adrenoceptor binding parameters in brain tissues of rats after the oral administration of prazosin and tamsulosin [34, 35]. Thus, it seems unlikely that these agents have any pharmacological effects on the CNS.

Muscarinic receptor binding in the bladder

Oxybutynin was widely used to treat OAB, but its use was often limited by systemic side effects, such as dry mouth, blurred vision, constipation and tachycardia, which appear frequently in patients receiving oral oxybutynin [42]. To overcome these drawbacks, a controlled release dosage form and a transdermal therapeutic system were developed and clinically used, with the advantage of a lower incidence of adverse effects than the immediate release form in patients with OAB [43–45]. These clinical observations coincide with the ex vivo data that oral but not transdermal oxybutynin produced a long-lasting blockade of muscarinic receptors in the salivary gland [46]. Further more, the antagonism by oral oxybutynin, unlike transdermal oxybutynin, was characterized by a marked suppression of the maximal response of pilocarpine-induced salivation. Taking account of the fact that muscarinic receptor binding affinity of the active metabolite, N-desethyl-oxybutynin (DEOB), was greater in the salivary gland than in the bladder [47, 48], the formation and tissue accumulation of a significant amount of DEOB may be responsible for the long-lasting occupation of exocrine receptors after oral oxybutynin treatment. Additionally, it is assumed that pharmacokinetic characteristics of the controlled release form of oxybutynin, such as substantially less fluctuation in the plasma oxybutynin levels, bring about a significant difference in exocrine receptor binding characteristics from those of oral oxybutynin.

Tolterodine, a potent antimuscarinic agent, displays favourable tissue selectivity for the urinary bladder compared with the salivary glands [49–51]. Tolterodine is extensively metabolized in the liver to form an active metabolite, 5-hydroxymethyl metabolite (5-HM) [50, 51]. In in vitro experiments, tolterodine, 5-HM and solifenacin displayed concentration-dependent binding of muscarinic receptors in the bladder and salivary gland [52, 53]. Oral administration of tolterodine and solifenacin compared with oxybutynin resulted in relatively slower and longer-
lasts for a longer time in the bladder compared to the salivary gland [23, 52–54]. Furthermore, significant receptor binding activity by a lower dose of tolterodine was also observed only in the bladder. Therefore, oral tolterodine binds more selectively to muscarinic receptors in the bladder than in the salivary gland. Tolterodine was significantly weaker than oxybutynin in inhibiting pilocarpine-induced salivation in mice [53]. Considering that most of the administered dose in mice receiving oral [14C]tolterodine was preferentially distributed to organs such as the urinary bladder, liver, and kidney [55], the plausible mechanism for the bladder receptor selectivity by oral tolterodine may be closely associated with the preferential distribution of this drug and 5-HM in the tissue.

Propiverine is an effective drug for urinary incontinence because of detrusor overactivity, with a moderate incidence of adverse events [56, 57]. The *in vitro* muscarinic receptor binding activity of propiverine and its N-oxide metabolite (DP-P-4(N→O)) is roughly equipotent. Following oral administration of propiverine, there was relatively selective and longer-lasting binding of muscarinic receptors in the bladder compared with the salivary gland [58–60]. A high concentration of DP-P-4(N→O) was detected in the bladder tissue of rats orally administered propiverine, indicating a specific distribution in the target organ. The intravesical instillation of DP-P-4(N→O) produced significant muscarinic receptor binding in the rat bladder [60]. Thus, DP-P-4(N→O) may contribute greatly to the relatively selective and long-lasting occupation of bladder muscarinic receptors after the oral administration of propiverine.

Imidafenacin, a potent M1- and M3-subtype-selective antagonist, is now used clinically in Japan for the treatment of OAB [61, 62]. Clinical studies indicated a favorable efficacy-to-side-effect ratio of imidafenacin in patients with OAB [63, 64]. The preferential muscarinic receptor binding in the bladder over the salivary gland, heart, colon, lung, and brain of this agent is demonstrated by *ex vivo* receptor binding (Figure 4) [65]. The oral administration of imidafenacin at low doses caused a more selective and longer-lasting binding to muscarinic receptors in the bladder than in other tissues. Pharmacokinetic data show that the orally administered imidafenacin distributed at a higher concentration in the bladder than in the serum or submaxillary gland of rats. Further more, a significant level of imidafenacin was detected in the urine of rats treated orally with this agent. The intravesical instillation of imidafenacin resulted in significant binding of bladder muscarinic receptors. Thus, it is likely that imidafenacin administered orally distributes predominantly to the bladder and binds, in a more selective and longer-lasting manner, to muscarinic receptors in this tissue than in other tissues, such as the submaxillary gland. Imidafenacin excreted in urine is assumed to play an important role in the pharmacokinetic and pharmacological selectivity.

Assessment of brain muscarinic receptor binding by positron emission tomography

Positron emission tomography (PET) is an imaging technique to monitor the delivery of tracers labelled with positron emitters. A variety of probes have been labelled to measure biochemical and physiological parameters in the CNS, such as glucose and oxygen metabolism, protein synthesis, blood flow and neurotransmitter receptors. A higher resolution PET system was applied for the *in vivo* imaging of neuronal activation and plasticity in the rodent brain [66, 67]. PET is a powerful noninvasive technique that can be used to examine the distribution and receptor binding of radioligands or novel drugs in the CNS [68, 69]. When the receptor binding of drugs is assessed by use of PET, several factors, such as the endogenous neurotransmitter and/or the metabolism of radioligands, should be carefully taken into account, otherwise serious mistakes could be arise [68]. Therefore, PET results should be always assessed in the combination with data obtained by other analytical methods, such as *ex vivo* binding assay and pharmacological procedures. Unfortunately, receptor binding measurement by PET in most peripheral tissues, including the lower urinary tract, is not currently successful.

The *in vivo* imaging of brain receptors by autoradiography (ARG) and PET allows the precise localization of muscarinic receptors and the pharmacological characterization of muscarinic receptor antagonists. The testing of muscarinic receptor antagonists for side effects on the CNS is important. Yoshida et al. [70] noninvasively characterized muscarinic receptor occupancy in the rat brain after the systemic injection of oxybutynin, darifenacin and imidafenacin using PET, comparing the results with those of *in vivo* ARG and *ex vivo* radioreceptor assays. In the PET study using rats, the intravenous injection of oxybutynin, but not darifenacin or imidafenacin, at pharmacological doses decreased the binding potential of (+)N-[11C]methyl-3-piperidyl benzilate ([11C](+)-3-MPB), a PET ligand of muscarinic receptors, in the cerebral cortex and corpus striatum in a dose-dependent manner (Figure 5) [70]. Likewise, in the receptor ARG analysis, oxybutynin decreased the binding of [11C](+)-3-MPB in each region of the brain in a dose-dependent manner, whereas darifenacin and imidafenacin had little effect (Figure 6). Also, a PET study in conscious rhesus monkeys showed that the oral administration of oxybutynin at clinically relevant doses resulted in significant (40–60%) occupancy of muscarinic receptors in the brain (SY, YI and HT, unpublished observations). In agreement with the PET results, short-term and chronic administration of oxybutynin in elderly subjects resulted in mild cognitive dysfunction [71, 72]. Consequently, the results obtained by ARG and PET [70, 73] confirm *ex vivo* binding data for oxybutynin, darifenacin and imidafenacin on muscarinic receptors [23, 65, 74] and seem to be
responsible for clinical observations indicating that darifenacin and imidafenacin might have fewer adverse effects on the CNS than oxybutynin [63, 71, 72, 75].

The intravenous injection of propiverine, tolterodine and solifenacin significantly decreased in vivo specific binding of [11C](+)-3-MPB in each region of the brain in rats in a dose-dependent manner [73]. Their estimated RO50 values (doses at which a half-maximal receptor occupancy is obtained) are similar to i.v. doses inhibiting learning/memory behaviour in rats [76]. Thus, these values could be regarded as an index of CNS pharmacological effects following the blockade of brain muscarinic receptors. The dose ratios (RO50/ID50) of muscarinic receptor antagonists for brain receptor occupancy to the inhibitory potency of increases in intravesical pressure, which reflect in vivo pharmacological selectivity for the urinary bladder over the brain, are relatively large for solifenacin, tolterodine and propiverine compared with oxybutynin. In fact, the incidence of CNS side effects of tolterodine was lower than that of oxybutynin in patients with OAB [77, 78]. Thus, the selectivity for the urinary bladder over the brain seems to be relatively high for new-generation muscarinic receptor

Figure 4
Muscarinic receptor binding (increase in the dissociation constant, Kd, for specific [3H]NMS binding) in the bladder, submaxillary gland, heart, colon, lung and brain of rats 1–12 h after the oral administration of imidafenacin [0.25 (A), 0.50 (B) or 2.0 mg kg⁻¹ (C)]. Control (■); 1 h (■); 3 h (■); 6 h (■); 9 h (■); 12 h (■)
antagonists, such as imidafenacin, suggesting a low likelihood of CNS side effects at pharmacological doses to treat OAB.

Muscarinic receptor antagonists must first cross the BBB to distribute into the brain and to occupy the CNS receptors. The observed difference among muscarinic receptor antagonists in the degree of uptake and binding to muscarinic receptors in the brain may depend on their ability to permeate the BBB. The passive penetration of the BBB is dependent principally on physicochemical properties [77], and thus its small size and molecular characteristics (high lipophilicity and neutral polarity) make oxybutynin likely to cross the BBB. In contrast, both darifenacin and imidafenacin have moderate polarity and low lipophilicity, suggestive of lower permeability. In addition, darifenacin is considered a substrate of P-glycoprotein, an active-transport system that carries this agent back across the BBB [79].

The muscarinic receptor subtype selectivity of muscarinic receptor antagonists may also be involved in the risk of side effects. Although all five muscarinic receptor subtypes are expressed in the CNS [80], the M1 receptor, in particular, is considered to play a crucial role in modulating cognitive function [81]. Oxybutynin and imidafenacin are selective for both M1 and M3 receptors, while darifenacin is selective of M3 receptors [22, 61, 82]. Thus the side effect of oxybutynin on the CNS during OAB treatment may be attributed to the selectivity for the M1 receptor in addition

Figure 5
(A) Typical positron emission tomography images fused with computed tomography images in the brain of rats injected intravenously with [11C](+)-3-MPB. The colour coding represents linear pseudocolouring of phosphor imager signal units (% of maximum). This modified figure was reproduced from reference [70] with permission

Br J Clin Pharmacol / 72:2 / 211
to the ability to permeate the BBB. In contrast, imidafenacin exerts little influence on cognitive function [62] and on brain muscarinic receptor binding [70], possibly because of the insignificant distribution of imidafenacin in the CNS.

**Comparison of in vivo and ex vivo receptor binding assay**

For in vivo study with radioligands, rats and mice can generally be used, but mice may be more convenient for the in vivo measurement of the distribution and receptor binding in tissues by use of a small amount of the ligand. In contrast, rats may be useful for the ex vivo receptor binding assay of novel drugs after systemic administration because of the larger amount of tissues. The tissue distribution and direct receptor binding in vivo could be estimated by the determination of radioactivity in various tissues after the i.v. injection of a well-characterized radioligand for a certain receptor (e.g. [3H]NMS for muscarinic receptors) or radiolabelled form of a novel drug. The technique using PET could also monitor the delivery and receptor binding of tracer labelled with positron emitters. Using these methods, we could assess the in vivo receptor binding.
activity and selectivity of nonlabelled novel drugs pre-treated with high sensitivity by the competition with radioligand. However, in this case, the definition of non-specific binding and the pharmacokinetics, such as the metabolism and protein binding of the radioligand, should be cautiously considered for the exact estimation of the receptor binding. Furthermore, it should be kept in mind that such in vivo methodology represents receptor binding characteristics with only a trace (not a pharmacological) dose of the radioligand.

The ex vivo binding assay using a well-characterized (standard) radioligand in tissues removed after the systemic administration of nonlabelled novel drugs allows investigation of the receptor binding characteristics (extent, duration, etc.) at pharmacological doses. In fact, the competitive or noncompetitive mode of receptor binding of novel drugs could be predicted by the changes of binding parameters (dissociation constant or maximal binding sites) of radioligand. However, there is a possibility that ex vivo study underestimates the amount of receptor binding of the administered drugs in tissues because of some dissociation of the receptor-bound drugs from the receptor sites during the preparation of tissue homogenates. In the ex vivo study compared with in vivo study, the dissociation of the receptor-bound drugs should be cautious. Most importantly, the precision of in vivo and ex vivo receptor binding data could be confirmed by the consistency with functional data on drugs determined by in vivo pharmacological methods, particularly in terms of pharmacological specificity, such as effective dosages, time course, duration and tissue selectivity. In fact, essentially similar results are obtained in the in vivo and ex vivo measurement of receptor binding of both α1-adrenoceptor antagonists and muscarinic receptor antagonists used to treat lower urinary tract symptoms, and they generally corroborate pharmacological results [20–23, 25–28, 34, 35, 46, 48, 52–54, 59, 60, 65, 70, 73, 74, 82, 83].

**Future directions**

The in vivo drug–receptor binding may be largely influenced by physiological factors, such as the intrinsic activity of nervous and hormonal systems. It is speculated that pathological conditions such as BPH and OAB affect in vivo binding of α1-adrenoceptor antagonists and muscarinic receptor antagonists. Significant alterations of pharmacological receptors in the lower urinary tract of experimental animal models with lower urinary tract symptoms were revealed [84–87]. Also, there were significant alterations of central and peripheral muscarinic receptors in tissues of muscarinic receptor knockout mice [80, 88]. Muscarinic receptor subtype knockout mice may be useful to characterize the in vivo muscarinic receptor binding of muscarinic receptor antagonists, because muscarinic receptor binding in the bladder of M2 knockout mice was significantly greater for oral solifenacin than oral oxybutynin [83]. The extensive analysis of in vivo drug–receptor binding characteristics in pathological states such as BPH and OAB may be of great importance for the preclinical evaluation of therapeutic agents in lower urinary tract symptoms.

**Conclusions**

The in vivo and ex vivo techniques using ARG and PET to measure drug–receptor binding are useful in predicting the potency, organ selectivity and duration of action of drugs in relation to their pharmacokinetics. Such evaluations of drug–receptor binding reveal that adverse effects could be avoided by the use of relatively new α1-adrenoceptor antagonists and muscarinic receptor antagonists for the treatment of lower urinary tract symptoms. The comparative analysis of α1-adrenoceptor and muscarinic receptor binding characteristics in the lower urinary tract and other tissues after the systemic administration of therapeutic agents allows the rationale for their pharmacological characteristics from the integrated viewpoint of pharmacokinetics and pharmacodynamics. In fact, pharmacokinetic factors significantly influence drug–receptor binding in physiological and pathological conditions. Thus, the in vivo and ex vivo characterization of drug–receptor binding may introduce a third dimension to preclinical pharmacokinetics and pharmacodynamics, with potential application not only in urology, but also in other therapeutic areas.

**Competing Interests**

There are no competing interests to declare.

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**REFERENCES**


