THE PHARMACOLOGY OF SVT-40776, A NEW MUSCARINIC ANTAGONIST FOR THE TREATMENT OF URINARY INCONTINENCE

Memòria presentada per Carolina Salcedo i Roca per a l'obtenció del grau de Doctor per la Universitat Autònoma de Barcelona.

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Foto portada realitzada per Joan Cabellos. Detall muscle detrusor de ratoli dins un bany d'òrgans.

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C E R T I F I C A

Que la present tesi doctoral, presentada per Carolina Salcedo i Roca amb el títol *The pharmacology of SVT-40776, a new muscarinic antagonist for the treatment of urinary incontinence* ha estat realitzada sota la meva direcció.

I per què així consti als efectes oportuns, signo el present certificat en Barcelona, a ……..

Aprendre, per saber-se desprendre, vet aquí el vell secret
(LLuis Llach)

Al Nigel, per la seva paciència i comprensió incondicional

A l'Emma, l'Erik i la Clara

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INTRODUCTION

1. URINARY INCONTINENCE

1.1. Pathogenesis and Aetiology

Urinary incontinence (UI) is defined as a condition in which the involuntary loss of urine represents a social and/or hygienic problem (Andersson *et al.,* 2001). The overall incidence of UI increases progressively with age. There are three types of UI: urgency, stress and mixed UI.

We will focus the study on urgency incontinence, considered to be due to a hyperactivity of the bladder, thereby called Overactive Bladder UI (OAB). This is a common clinical problem which may originate from dysfunction of the peripheral or central nervous pathways, the urotehlium, the smooth muscle and other tissue components. Unlike most other autonomic control mechanisms, conscious control plays a major role in normal LUT (Lower Urinary Tract) function, allowing a discrete voiding under environmentally appropriate conditions. When conscious control of the parasympathetic micturition reflex is altered, symptoms of OAB arise, creating a serious health problem commonly associated with a high psychological and social impact.

The ICS (International Continence Society) definition for OAB (Abrams *et al.,*, 2002) is:

“urgency, with or without urge incontinence, usually with frequency and nocturia can be described as OAB syndrome”.

OAB involves symptoms of urgency, frequency and even leakage in the most serious cases. It is the most common cause of urinary incontinence. OAB is characterized by involuntary detrusor contractions that may occur spontaneously or may be provoked, such as by rapid filling, alterations of posture, coughing, walking, or jumping, all while the patient is attempting to suppress them (Hampel *et al.*, 1997). The involuntary detrusor (bladder muscle) contractions are often symptomatic, associated with a desire to void, urgency, frequency, nocturia, and leakage (Hampel *et al.*, 1997; Bo *et al.*, 2000; Weiss, 1998).

Myogenic aetiology: in same cases the symptoms of OAB might be due to disorders on smooth muscle tone. Bladder tissue from these patients shows distinct features at the smooth muscle level predisposing them to unstable contractions. The loss of normal excitatory neural input results in increasing signalling between smooth muscle cells, leading to a state of overactivity. Acute sensitivity to agonist increases in gap junctions and enhances electrical coupling between smooth muscle cells. This fact induces the existence of widespread despolarization signals sufficient to cause spontaneous muscle activity resulting in increased intravesical pressure (Yoshimura *et al.*, 2002). In this case it is also called detrusor instability. The causes are often idiopathic.

Neurogenic aetiology or detrusor hyperreflexia: the symptoms of OAB may be also triggered by neurogenic defects or trauma (Alzheimer, Parkinson, multiple sclerosis, spinal cord injury and stroke). In these patients a loss of inhibition of the sacral reflex (see Chapter 2.2) through the pelvic nerve modifies reflex regulation of both bladder and urethral function leading to bladder hyperreflexia (Yoshimura *et al.*, 2002).

Urinary incontinence affects an estimated 33 million U.S. citizens (16.5% over 18 years old), a prevalence even higher to that of other chronic diseases, including asthma or angina. It affects the same ratio (over 16 %) in Europe. The prevalence among persons 15 to 64 years of age is 10% to 25% for women and 1.5% to 6% for men. In a survey of patients aged 50 years and older, 27.6% of women and 10.8% of men reported urinary incontinence (Thomas *et al.* 1980; Steward *et al.* 2003, Irwin *et al.*, 2006). OAB is the most common type of established incontinence; it accounts for about 65% of cases (Resnick *et al.*, 1985; Rousseau *et al.*, 1992).

Table 1. UK comparative incidence of most prevalence pathologies

UK COMPARATIVE INCIDENCE DATA	
OAB	19%
ASTHMA	7%
ANGINA	2%
DIABETES	2%

Urinary incontinence is under-reported and under-diagnosed. Even though the overwhelming majority of incontinent patients are troubled by the condition, they do not report it to their physician in many cases (Diokno *et al.*, 1995). It is

estimated that less than half of community-based persons with urinary incontinence consult a health care provider about the problem. The major reasons for lack of reporting are assumed to be embarrassment and the perception of social stigma. Another reason is the belief that it is an inevitable and untreatable consequence of aging. Most patients think that incontinence is part of normal life, whereas others judge their incontinence not to be severe or frequent enough to require treatment (Hampel *et al.*, 1997) One study found that one-third of women with urinary incontinence had never mentioned their symptoms to a physician (Mitteness, 1987). Thus, it seems clear that many of the people affected by OAB symptoms do not seek help from heath care professionals (Nabi *et al.*, 2006).

1.2. Current pharmacological treatments

Drugs focus on suppressing the micturition reflex (see paragraph 2.2.5.) could be useful for treating OAB. Six categories can be defined depending on the site of action (Table 2) (Fraser *et al.*, 2003). In this study we will focus on the treatments related to the bladder smooth muscle neuroeffector junction. In normal as well as in abnormal micturition, contraction of the detrusor smooth muscle is mediated by acetylcholine (ACh), which is released from postganglionic parasympathetic neurons and acts at muscarinic receptors on detrusor smooth muscle (see paragraph 3.1). Thus, muscarinic receptors are primarily responsible for neurologically-induced excitations of smooth muscle cells (Abrams *et al.*, 2006).

Five subtypes of muscarinic receptor types have been identified (M_1-M_5). M_1 receptors are located primarily in neural tissue, they are abundant in brain and in peripheral sympathetic ganglia; M_2 are located in cardiac and detrusor smooth muscle; M_3 are located in the smooth muscle (detrusor, intestinal, pulmonary), intestinal tract (Uchiyama *et al.*, 2004) and salivary and other excretory glands; M_4 receptors have site selectivity to the cerebral cortex, striatum and lung; and M_5 are located in the substantia nigra (Chappel *et al.*, 2000) cerebral vessels and, probably, salivary glands (Yeomans *et al.*, 1999; Chappel *et al.*, 2000) M_2 receptors are the predominant population in human detrusor (80%) (Mansfield *et al.*, 2005) while the M_3 receptors are the main functional mediators of acetylcholine action increasing detrusor contractility (Chappel *et al.*, 2000) M_3 receptors directly mediate bladder contraction, while M_2 receptors inhibit the sympathic mediated detrusor relaxation. M_2 is the only muscarinic receptor

mediating the vagal tone of the heart, thereby modulating the cardiac inotropism and cronotropism (Andersson *et al.*, 2007). Some results suggest that, in addition to the predominant M_3 receptors, M_1, M_4 and M_5 can have a partial role in regulating salivation (Andersson, 2002).

As M_3 muscarinic receptor has been found in the detrusor muscle as the major responsible receptor for its contraction, cholinergic antagonists, by interfering with its action, reduce detrusor activity and thus are effective in treating OAB. Several studies have supported that antimuscarinics can depress involuntary bladder contraction (Low *et al.*, 1977; Cardozo *et al.*, 1979; Blaivas *et al.*, 1980). Thus, antimuscarinics are the most widely used treatment for UI (Anderssson *et al.*, 1999; Nabi *et al.*, 2006). However, the current treatments lack selectivity for the bladder and effects on other organ systems may result in side effects which limit their usefulness. The most relevant side effects are related with blurred vision, dry mouth, constipation, (M_3-receptor related effects). Cognitive dysfunction, memory loss and attention defit (M_1-receptor related effects) and cardiovascular, palpitations and taquicardia effects (M_2-receptor related effects). Therefore, extensive research has been produced on trying to find more selective compounds, in both, tissue selectivity (bladder *vs.* other organs) and receptor selectivity (M_3 *vs.* other receptors, especially M_2). Muscarinic receptor role in bladder function is extensively described in paragraph 3.1.

Worldwide there are only seven drugs, all antimuscarinics, currently marketed for the treatment of OAB (see Table 2): oxybutynin, tolterodine, propiverine, fesoterodine, trospium, darifenacin and solifenacin. Each one has demonstrated efficacy for the treatment of OAB symptoms but their pharmacokinetic and adverse event profiles differ.

Table 2. Current anticholinergic treatments for OAB

MOLECULE	BRAND	MUSCARINIC ANTAGONISM
Oxybutynin	Ditropan	Modest, non-pharmacologically relevant selectivity for M3
Oxybutynin XL (Extended release)	Ditropan XL 2002	
Oxybutynin TDS (Transdermal patch)	Oxytrol	
Tolterodine	Detrol	Non selective
Tolterodine-LA (Extended release)	Detrol LA	
Trospium	Sanctura	Non selective
Trospium- XR (Extended release)	Sanctura	Non-selective
Solifenacin	Vesicare	Modest, non- pharmacologically relevant selectivity for M3
Darifenacin	Emselex Enablex	Moderate M3 selectivity
Fesoterodine	Toviaz	Non-selective
Propiverine	Detrunorm	Amdipharm

We will only review the most relevant treatments in terms of market importance and strategy followed. Thus, we will describe exclusively 4 drugs. The two classical muscarinic antagonists, oxybutynin and tolterodine, that concentrate more than 95% of the OAB world market, and solifenacin and darifenacin,

launched recently (2004 and 2005 respectively) that claim increased M_2 *vs.* M_3 selectivity.

Oxybutynin has both anticholinergic and direct smooth muscle relaxant properties. It is likely that the primary action is via ACh antagonism (Chappel *et al.*, 2000). It is a non-selective muscarinic antagonist although a non-relevant degree of selectivity for M_3 and M_1 receptors over other muscarinic subtypes has been described from radioligand binding studies (Chappel *et al.*, 2000). It has high affinity for the salivary parotid glands and the central nervous system. It is rapidly absorbed from the gastrointestinal tract when given orally but it is extensively metabolized through the cytochrome P450 system in the liver, specifically the 3A4 enzymes. Only negligible amounts of the parent compound are excreted renally. Its half-life is from 2 to 5 hours. Its main active metabolite, N-desethyloxybutynin, has also significant antimuscarinic activity. Following oral administration of oxybutynin, levels of the metabolite are some 6-fold higher compared with the parent oxybutynin. It is thought that the active metabolite, along with the parent compound, cause the anticholinergic adverse effects (Chappel *et al.*, 2000). Adverse effects are antimuscarinic and dose related. Side effects can be particularly disturbing to patients, and may be of sufficient severity to result in poor compliance with therapy (Thuroff *et al.*, 1991; Ouslander *et al.*, 1988; Kelleher *et al.*, 1994). Dose-dependent dry mouth is the most frequently reported side effect and is often cited as a major reason that patients discontinue oxybutynin therapy (Burgio *et al.*, 1997; Jacquetin, 1997; Lam *et al.*, 2007). Other side effects include constipation, tachycardia, palpitations, somnolence, dizziness, restlessness, blurred vision, hallucinations, insomnia, and impotence. In order to minimize the side effects, two new formulations were developed, and extended release (XL) tablet taken once daily and a transdermal patch applied twice weekly. However, when comparing these formulations with its main competitor, tolterodine ER (see next paragraph) in different clinical trials (OBJECT, OPERA, ACET studies) oxybutynin was never better than tolterodine on side effects (Lam *et al.*, 2007).

Tolterodine and its major active 5-hydroxymethyl metabolite (DD01) are muscarinic receptor antagonists that are claimed to be more active in the bladder than the salivary glands (Appell, 1997). Tolterodine exhibits a preferential inhibition of M_2 muscarinic receptors although it also shows relevant affinities for

the whole range of subtypes of muscarinic receptors. Tolterodine is rapidly absorbed from the gastrointestinal tract. It is metabolized by cytochrome P2D6 to DD01 and by CYP3A4 in poor metabolizers. The half-life of tolterodine is 2 to 3 hours and 3 to 4 hours for DD01. Both unchanged drug and metabolites are excreted in the urine (Tolterodine: Medical letter, 1998). Tolterodine reduces the number of micturitions in a 24-hour period compared to placebo. However, the reduction in the number of incontinent episodes was not statistically superior to placebo (Tolterodine: Drug Expert Reports, 1998). Differences in adverse effects between tolterodine and oxybutynin have been demonstrated in several preclinical studies, including *in vivo* studies (Appell, 1997) It appears from a review of four studies of tolterodine in 12-week trials that the optimal dose is 2 mg given twice daily; tolterodine has similar efficacy when compared with oxybutynin; and tolterodine is associated with a lower incidence of side effects, especially dry mouth, when compared to immediate-release oxybutynin (Tolterodine: Drug Expert Reports, 1998; Lam *et al.* 2007). Tolterodine, as well as oxybutynin, has developed a new formulation focus on reducing side effects, an extended release (ER) capsule taken once daily. Several clinical trials have been carried out with the new formulation comparing efficacy and tolerability with oxybutynin-XL and oxybutynin transdermal.

The OBJECT (Overactive Bladder: Judging Effective Control and Treatment) study was a prospective, randomized, double blind, parallel.group study that compared the efficacy and tolerability of oxybutynin-XL (10 mg once daily) to tolterodine (2 mg twice daily). Results showed that oxybutynin-XL was significantly more effective in weekly urge incontinences, total incontinences and micturition frequency episodes. Adverse events were similar, with dry mouth being reported most commonly (Appell *et al.*, 2001).

The OPERA (Overactive Bladder: Performance of Extended Release Agents) trial was a 12-weeks double blind study comparing oxybutynin–XL (10 mg once daily) vs tolterodine–ER (4 mg once daily). Results showed similar improvements in weekly urge incontinence as well as significant greater reduction in micturition frequency in the oxybutynin group. Tolerability was similar with the exception of dry mouth which was more commonly reported in oxybutynin group (Diokno *et al.*, 2003).

The ACET (Antimuscarinic Clinical Effectiveness Trial) study consisted of two trials; in one, patients with overactive bladder were randomized to 8 weeks of open-label treatment with either 2 mg or 4 mg of once-daily tolterodine-ER (TER) and in the other to 5 mg or 10 mg of oxybutynin-XL (OER). Dry mouth was dose-dependent with both agents, although differences between doses only reached statistical significance in the oxybutynin trial (OER 5 mg vs OER 10 mg; $p = 0.05$). Patients treated with TER 4 mg reported a significantly lower severity of dry mouth compared with OER 10 mg. The greater efficacy and tolerability of tolterodine ER 4 mg suggested improved clinical effectiveness compared with oxybutynin-XL 10 mg (Sussman *et al.*, 2002).

Another study compared the efficacy and safety of transdermal oxybutynin to oral tolterodine-ER. This 12-weeks study resulted in lower and similar efficacy for both compounds. Moreover, adverse effects were not abolished with the transdermal patch so dry mouth still appeared with lower indidence than tolterodine, apart from application site pruritus for the patch (Dmochowski *et al.*, 2003)

In conclusion, efficacy had been widely shown, to a greater or lesser extent, in all clinical trials, as well as constant adverse events associated with tolterodine that include dry mouth, dyspepsia, headache, constipation, dry eyes and cardiac effects (Appell, 1997; Appell *et al.*, 2001; Sussman *et al.*, 2002; Diokno *et al.*, 2003; Olshansky *et al.*, 2006; Lam *et al.*, 2007).

A safe cardiac profile is especially relevant for senior patients, often chronically polymedicated (Andersson *et al.*, 2007). An increase in heart rate is a well-known adverse effect of non selective antimuscarinic agents, which may become prominent at high doses. Unfortunately, the cardiovascular parameters have been hardly published. Recent evidence come from a meta-analysis of available randomized trials to evaluate the efficacy and safety of tiotropium, a non-selective muscarinic antagonist that was developed for the treatment of chronic obstructive pulmonary disease (COPD). Among the adverse events reported, the authors point out that the frequency of arrhythmias was significantly higher with tiotropium than with placebo (Barr *et al.*, 2006). Moreover, the effects of darifenacin and tolterodine on heart rate have been recently evaluated in patients with overactive bladder (Olshansky *et al.*, 2006). Tolterodine significantly increased heart rate in comparison with darifenacin and placebo and the proportion of patients with heart

rate increases of ≥5 or ≥10 bpm were higher with tolterodine than either placebo or darifenacin. These findings certainly support our hypothesis that selective M_3 *vs*. M_2 receptor antagonists may provide a safer cardiovascular profile. Thus, the main objective for the development of extended release formulations of oxybutynin and tolterodine in order to achieve a once a day dose regimen was to increase compliance but specially to decrease side effects.

However, oxybutynin-XL and the transdermal patch have been reported also to produce cardiovascular effects on patients. The XL formulation produced a 2% to <5% of patients hypertension, palpitations and vasodilation (Ditropan, 2003). The transdermal formulation did produce cardiac arrhythmia reported with overdosage (FDA, 2006).

Propiverine do also produce cardiovascular effects including heart rate increase that have been reported in two clinical trials (Dorschner *et al.*, 2000; Jabs *et al.*, 2001).

Darifenacin and solifenacin are the new selective muscarinic M_3 receptor antagonists developed specifically for the OAB treatment. Both drugs were developed following the objective of receptor selectivity. They have shown to be a safer, but not a more effective, treatment for the OAB than current treatments.

Darifenacin shows an *in vitro* selectivity for human cloned muscarinic M_3 receptor relative to M_1, M_2, M_4 and M_5. Theoretically it may be argued that M_3 *vs.* M_1 receptor selectivity may provide an advantage over non-selective agents since both M_3 and M_1 receptors have been related to salivary secretion (Culp *et al.*, 1996). In an anaesthetised dog model, selectivity for the urinary bladder over the salivary gland had been demonstrated (Newgreen *et al.*, 1995; Wallis *et al.*, 1995). M_3 *vs.* M_1 selectivity may be also associated with low rate of cognitive impairment, M_1 related (Pavia *et al.*, 1998). M_3 *vs.* M_2 selectivity can provide a decreased effect on heart rate (M_2) and M_3 *vs.* M_5 selectivity has been suggested that can reduce impairment of visual accommodation (M_5) (Eglen *et al.*, 2000; Choppin *et al.*, 2000). Due to the short half life of darifenacin (darifenacin IR- t.i.d.), a controlled-release formulation was developed (darifenacin ER- doses of 7.5 and 15 mg). Four clinical 12-weeks studies evaluated the efficacy and safety of darifenacin ER 7.5 and 15 mg/day. In general the compound presented comparable efficacy with improved tolerability when compared with oxybutynin.

Darifenacin is devoid of central and cardiac effects in healthy volunteers but when administered in OAB patients, darifenacin ER produces significantly lower reduction in salivary flow than oxybutynin at therapeutically equivalent doses. Thus, it seems that again the safety margin is narrow and doses superior to 7.5 mg cannot be administered due to tolerability. The absence of cardiac effects is possibly a safety advantage, although still there are a great incidence on constipation and dry mouth (Lam *et al.*, 2007). Darifenacin was marketed in the US during the 2005, permitting to the urologist to choose and change the dose depending on the efficacy *vs.* side effects found in each specific case.

Solifenacin is a long acting muscarinic receptor antagonist also developed specifically for the treatment of OAB. It behaves similary to oxybutynin at human cloned receptors in that it has a modest (10-fold) selectivity for M_1 and M_2 receptor *vs.* M_3 (Ikeda *et al.*, 2002). However, unlike oxybutynin, solifenacin antagonised carbachol (CCh) -evoked increases of intracellular calcium in rat detrusor cells approximately 3.6-fold more potently than in submandibular cells. In anaesthetised rats, solifenacin had a bladder –to-salivary gland selectivity ratio of 3.7 –fold and 6.5 -fold, marginally superior to oxybutynin and darifenacin (0.97 and 1.0-fold respectively) (Ohtake *et al.*, 2004). Clinical data show efficacy in OAB patients. Solifenacin has a long elimination half-life that is further extended in patients with hepatic and renal impairment (Kuipers *et al.*, 2003; Smulders *et al.*, 2003). A placebo-controlled study compared the effects of two doses of solifenacin, 5 and 10 mg/day, demonstrated that while the higher dose produced a slightly greater reduction in daily micturitions, this was accompanied by a far greater incidence of dry mouth. Other clinical trials also show a high incidence in dry mouth in the higher dose, up to 32% dry mouth. A comparative study with tolterodine ER shows that solifenacin is more efficacious than tolterodine but both treatments cannot reduce dry mouth at any dose tested. Also, solifenacin presents a slightly higher incidence of blurred vision and constipation compared with tolterodine. However, more comparative trials with the new formulations of oxybutynin and tolterodine would be necessary to determine whether solifenacin represents an improvement over existing therapies (Hedge *et al.*, 2004). Solifenacin obtained the approval to be launched in the US and Europe in 2004 as a more safe, but not more efficacious, treatment for the OAB than previous treatments.

As commented previoussly, as the other antimuscarinic treatments marketed (trospium, fesoterodine and propiverine) do not improve the profiles described, we do not expand the information about them.

Thus, it can be said that OAB treatments still lack enough tolerability and efficacy to consider that this field has achieved maturity. The strategy of obtaining more selective agents is directed to increase the maximum tolerated dose yielding more effectiveness and patient compliance. Meanwhile, OAB is clearly an unmet clinical need.

2. LOWER URINARY TRACT

2.1. Anatomy

The function of the lower urinary tract (LUT) is to store and periodically release urine. In males the sexual function is also associated. The storage and micturition process requires the orchestration of reflexes involving both the sympathetic and parasympathetic components of the autonomic nervous system and somatic motor pathways. Key components include the end organs themselves - the bladder smooth muscle, the urethral smooth and striated muscles - as well as the peripheral and central nervous system neural circuit.

In both sexes, the urinary bladder lies in the anterior (ventral) part of the pelvic cavity. The proportion of the cavity that occupies dependens upon the volume of the fluid contained within the bladder lumen. The full bladder is approximately spherical in shape, becoming more tetrahedral in form as empting occurs (see Figure 1). When viewed from within, the mucosa lining the wall of the bladder presents three distinct apertures, namely the ureteric orifices and the internal urethral meatus. These lie relatively close to one another and delimit the trigonal region of the bladder. With the exception of the trigone, the bladder mucosa is rough in the indistended organ but became smoother as filling proceeds.

The bladder walls are formed by three layers, a peritoneal layer, a muscular (or detrusor muscle) and a mucosa layer, being this the inner one. Although the three layers contribute to the mechanical activity of the bladder, is the detrusor muscle actually the responsible for the bladder function. The detrusor is formed by individual smooth muscle cells, arranged in muscle bundles. In human detrusor the muscle bundles are large, often a few mm in diameter and composed of several smaller sub-bundles. These bundles are not clearly arranged in distinct layers, but run in all directions in the detrusor. Amongst the smooth muscle cells can be found cells having long dendritic processes extending parallel to the smooth muscle fibre. The orientation and interaction between the smooth muscle cells in the bladder are important, since this will determine how the bladder wall behaves and what effect activity in the cells will have on its shape and intraluminal pressure.

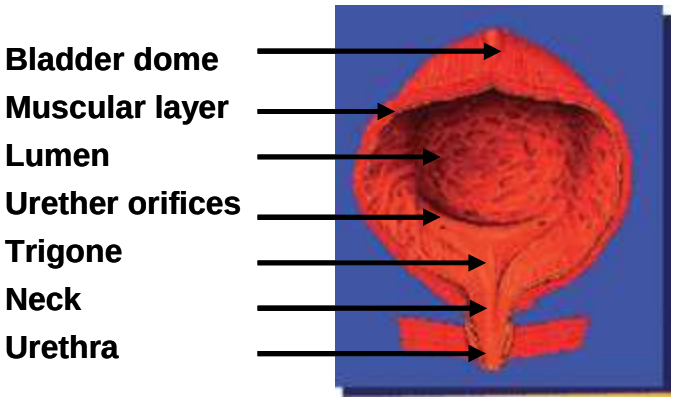


Figure 1. Scheme of a human bladder

When these smooth muscle cells contract, the pressure can increases up to 60 mmHg. This contraction will be the event before the bladder empties. The detrusor cells are fused permitting a low electrical resistance between them and a quick propagation of the action potential to the whole muscle. Thus, the whole bladder can contracts in a synchronised and simultaneous way.

The bladder neck (2 - 3 cm) is a detrusor muscle mixed with elastic tissue forming the internal sphincter. The contracted sphincter baseline tone maintains the bladder neck and the urethra closed, avoiding the bladder to empty until the pressure of the main organ over pass the critical level.

The urothelium is the bladder epithelium. It has been shown recently than the urothelium is much more than a classical barrier than separate urine from the extracellular fluid. It is also an active absorptive epithelium which absorbs sodium, using specific sodium channels in the urothelial cell membranes. The number of these channels is controlled by the level of AMPc in the cell and for the sodium balance in the animal. The urothelium is also a secretor tissue that secretes urinary proteins such as plasminogen activator and urokinase. The possibility that there may be some active control over the functional state of the urothelium has been recognised.

The urethra crosses the urogenital diaphragm which contains a muscle layer named bladder external sphincter, made up of striated muscle, different from the

body and neck bladder muscle, which are formed by smooth muscle cells. This sphincter is regulated by the voluntary nervous system and it is used to block the voiding even when the involuntary reflexes are giving the order to void.

2.2. Physiology

2.2.1. Supraspinal micturition reflex

Normal micturition is controlled by neural circuits in the brain and spinal cord that coordinate the activity of visceral smooth muscle in the urinary bladder and urethra with activity of striated muscle in the urethra sphincter. These circuits act as on-off switches to shift the lower urinary tract between two modes of operation: storage and elimination. Urine storage and release are subject to voluntary control, mediated by cerebral cortex (Figure 2). Thus, the neural control of the urinary tract is distinct from that of other visceral organs such the heart or intestine which are regulated exclusively by involuntary reflex mechanisms.

The reflex circuitry used by the micturition reflex includes:

- Parasympathetic pre-ganglionic neurons originating in the sacral parasympathetic nucleus (SNP).
- Somatic motoneurones innervating the external urinary sphincters (EUS) which appears to have reciprocal activity compared with SNP efferents neurons.
- Ascending and descending neurones that connect sacral primary afferents and the SNP with the Pontine Micturition Centre or Pons (PMC).
- Primary afferents from the lower urinary tract.

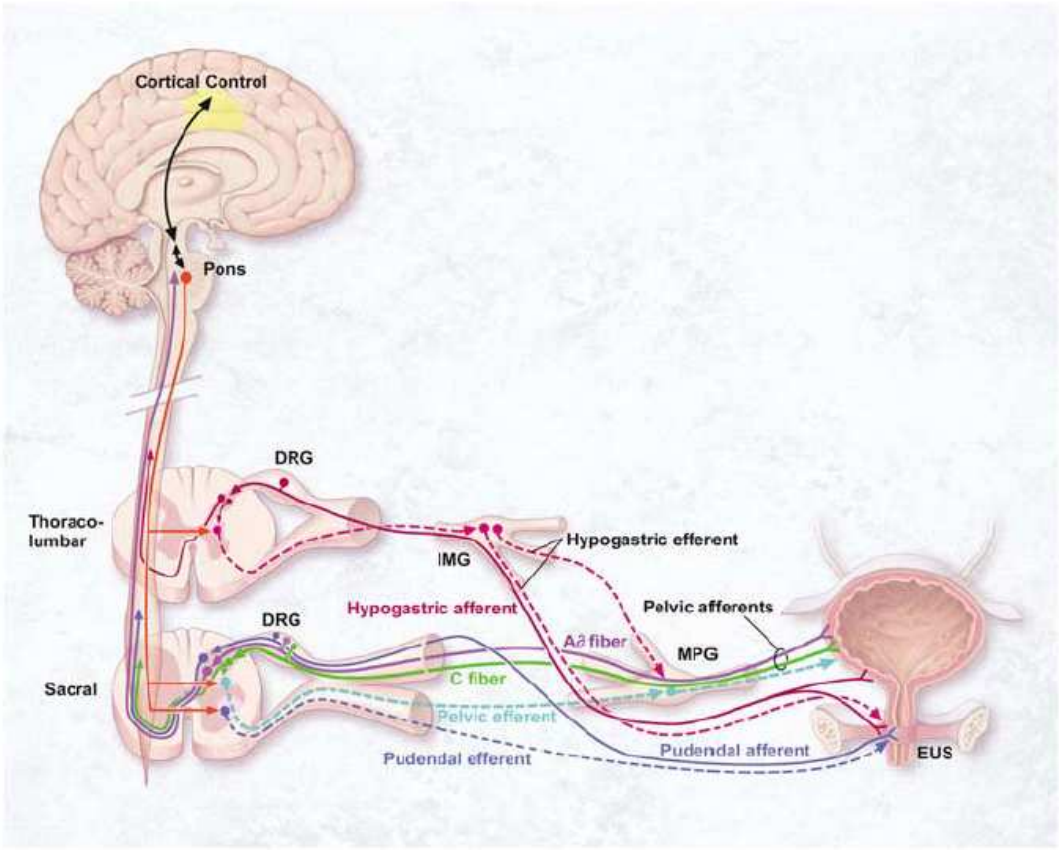


Figure 2. Schematic diagram of the neural circuits controlling continence and micturition.The majority of Adelta and C- afferents that innervate the urinary bladder and urethra are found in pelvic nerves, which also contain parasympathetic efferents originating from sacral spinal cord. The remaining bladder afferents are carried by hypogastric nerves, which also contain sympathetic efferents originating from the thoracolumbar spinal cord. Sacral somatic afferent and efferent innervation to the external urethral sphincter is via pudendal nerves. Under normal physiological conditions in adults, the micturition reflex is controlled predominantly by Adelta afferents, communicating via the spinal cord to supraspinal centres in the pons and cortex. Under pathophysiological conditions or with aging, spinal reflex mechanism mediated by C-fibres can became dominant- (Ford, A *et al*, 2006).

The micturition reflex is initiated by stretch receptors in the detrusor muscle which passively stretches during filling and actively contracts during micturition. The stretch receptors are terminals specializations of thinly myelinated Adelta afferents fibers that traverse the pelvic nerve to reach the spinal cord. The cell bodies of theses fibers are medium sized primary afferents neurons located in the sacral dorsal root ganglia (DRG).The central branches of these primary afferents neurons project along Lissauer's tract and the lateral edge of the dorsal horn to contact second-order neuron in the dorsal horn of the sacral spinal cord. Via a spinobulbospinal pathway, the second-order neurons in the sacral spinal cord project to the periacueductal grey matter of the brain, which in turn activates neurons in the PMC.

The PMC is located in the locus coeruleus. Neurons in the PMC project directly to bladder preganglionic neurons located in the lateral band region of the sacral parasympathetic nucleus and to interneurons in the sacral dorsal gray commissure. The axons of cholinergic sacral parasympathetic preganglionic neurons traverse the pelvic nerve to activate pelvic parasympathetic post-ganglionic neurons in the pelvic plexus via nicotinic cholinergic receptors. The cholinergic postganglionic neurons release acetylcholine (Ach) resulting in detrusor smooth muscle contraction via stimulation of M_2 and M_3 receptors.

Suprapontine control of the micturition reflex is crucial (Figure 3) for ensuring that micturition occurs within the proper behavioural and environmental conditions. Compromise of these suprapontine controls is thought to play a role in the aetiology of OAB that accompanies cerebrovascular stroke.

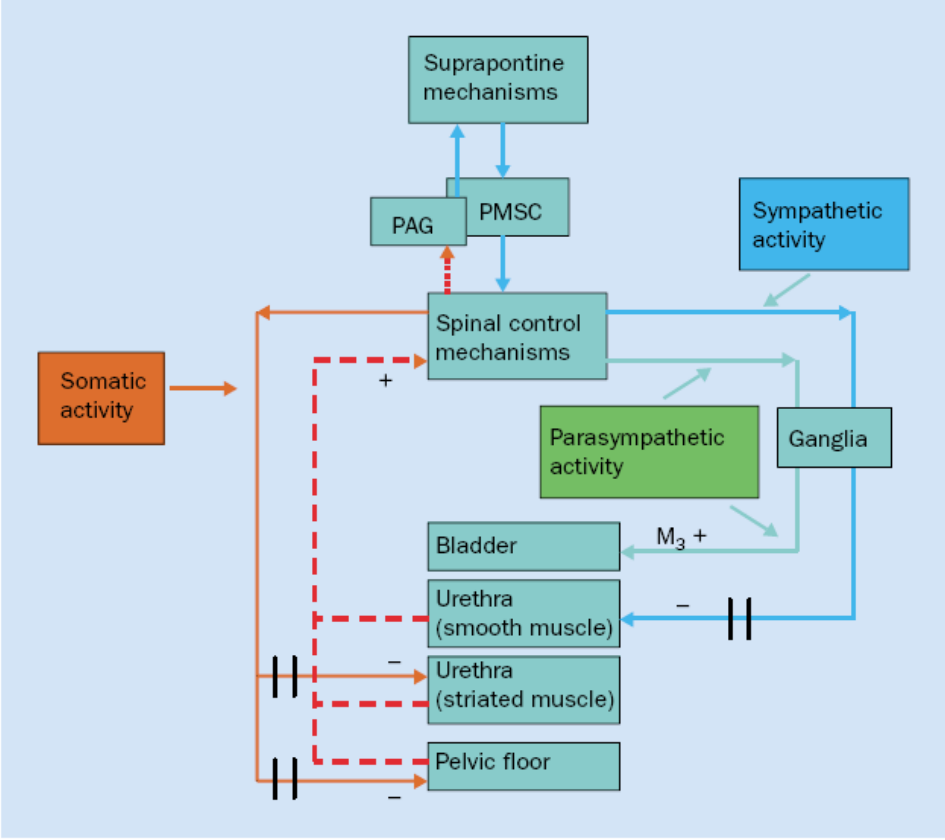


Figure 3. Volding reflexes involve supraspinal pathways and are under voluntary control. During bladder emptying, the spinal parasympathetic outflow is actived, leading to bladder contraction mainly via muscarinic receptors M_3. Simultaneously, the sympathetic outflow to urethral smooth muscle and the somatic outflow to urethral and pelvic-floor striated muscles are turned off, and the outflow region relaxes. PAG-periaqueductal grey; PMC: pontine micturition centre.

2.2.2. C-fiber spinal micturiton reflex

Under normal conditions the primary afferents C-fibers do not respond to bladder distension at normal bladder volumes. However, under pathological conditions, these C-fibers become responsive to bladder distension at volumes thresholds below those of Adelta fibers. This reflex is then organized within the sacral spinal cord region and do not depend on supraspinal communication to activate sacral parasympathetic preganglionic neurons. The parasympathetic efferent pathway of the C-fiber spinal reflex and the Adelta fibers is the same. Thus, sensitization of, and/or removal of inhibition from, the C-fibers reflex pathways are thought to contribute to the aetiology of OAB that accompanies benign prostatic hyperplasia, spinal cord trauma or disease and urinary tract infections.

2.2.3. Sympathetic Storage Reflex

This reflex is initiated also by bladder distension and activation of Adelta fibers of the pelvic nerve that activates sacral dorsal horn intreneurons. A polysynaptic pathway projects dorsal rostrally to activate efferents sympathetic preganglionic neurons, situated at L1-L3 levels. Their efferent axons travel to the inferior mesenteric ganglion (IMG) where they either synapse or continue along the hypogastric nerve to synapse in the pelvic plexus. Postganglionic sympathetic neurons release norepinephrine which facilitates urine storage by:

- stimulating β3 adrenergic receptors that relax bladder smooth muscle.
- stimulating α1 adrenergic receptors that contract urethral smooth muscle.
- stimulating α1 adrenergic receptors that inhibit ganglionic transmission.

During micturition such reflex is inhibited via supraspinal mechanisms to allow the bladder to contract and the urethra to relax.

2.2.4. Somatic Storage reflex

This reflex is initiated by activation of Adelta fibers of the pelvic nerve that, in turn, activate sacral dorsal horn interneurons. Polysynaptic connections allow the transmission to travel from the sacral dorsal horn to the sacral ventral horn, at S2-S4 levels. Efferent somatic urethral sphincter motor neurons are located in the lateral subdivision of Onuff's nucleus. The axons from these motor neurons traverse the pudendal nerve and release Ach which, in turn, activates nicotinic cholinergic receptors on striated muscle fibers of the urethra causing them to contract. During urine storage this pathway is tonically active and during micturition this reflex is strongly inhibited via spinal and supraspinal mechanisms to allow the urethral sphincter to relax and permit passage of urine though the urethra (Figure 4).

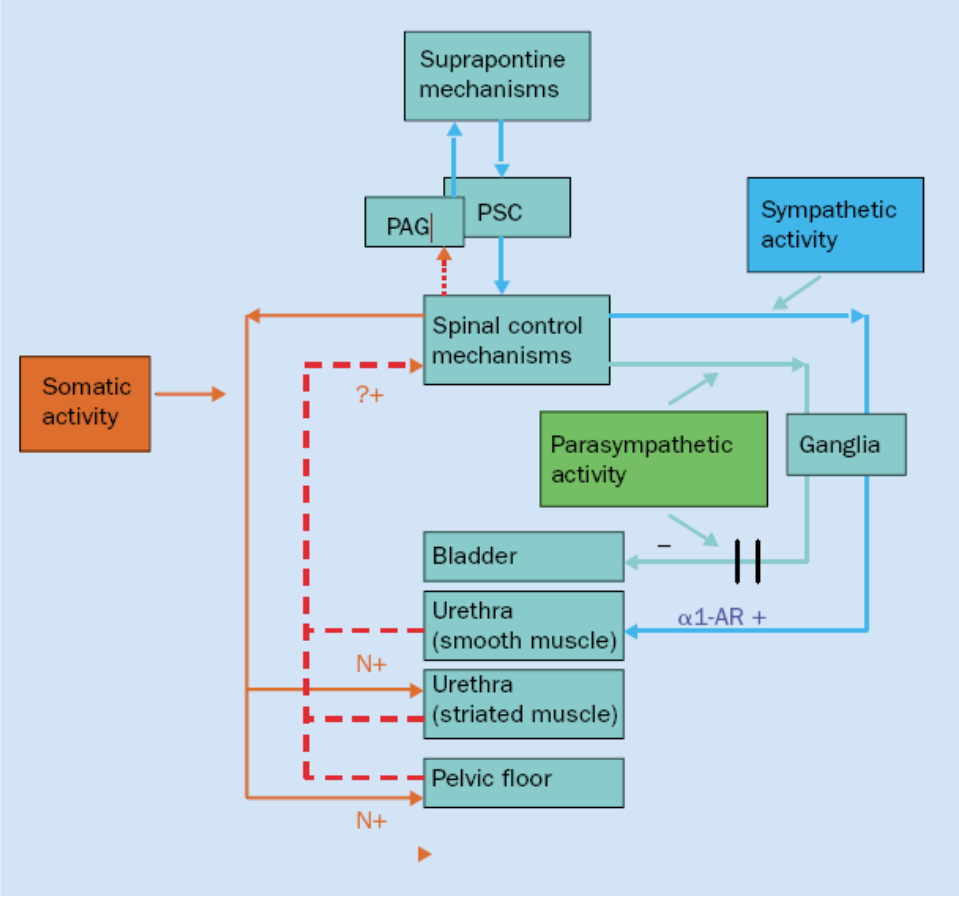


Figure 4. Storage reflexes are spinal reflexes under supraspinal control via periaqueductal grey (PAG) and the pontine storage centre (PSC). During filling, there is continous and increasing afferent activity from the bladder. There is no spinal parasympathetic outflow that can contract the detrusor. The sympathetic ouflow to urethral smooth muscle (α1-adrenoceptors [α1-AR]) and the somatic outflow to urethral and pelvic-floow striated muscles (nicotinic receptors [N]) keep the outflow region closed. Whether or not the sympathetic innervation to the bladder (via

β3-AR) contributes to bladder relaxation during filling in human beings is unclear. The transmitters involved in the spinal control of afferent activity have not been identified.

2.2.5. Sites of drug action for inhibition of micturition reflexes

Drugs suppresing the micturition reflex could be useful for treating OAB. Summarising what has been described in this Chapter, 6 targets can be defined(see Table 3). Therapeutic agents that suppress action potential initiation and/or propagation along primary afferent fibers, through manipulation of ion channels or G protein coupled receptors (GPCR) would be expected to increase the volume threshold for activation of the micturition reflex and thus reduce bladder overactivity and urgency. Once the action potential from the primary afferent fiber reaches its spinal synaptic terminal, an influx of calcium is necessary to cause fusion of the synaptic vesicles with the plasma membrane and subsequent release of the primary afferent neurotransmitters. This work has been focus in this first level of action, so interfering on the GPCR in order to block the micturition reflex. Thus we are not going to be extensive in the other possible mechanisms but we have listed all on Table 3

Table 3. Sites and targets for inhibition of the micturition reflex

SITE	TARGET	FUNCTION
Primary Afferent Neurons	Na channels	+ pe
	VR_1	+ pe
	P_2X_3	+ pe
	NK_2	+ pe
	NO	- pe
	K channels	- pe
Sacral Dorsal Horn	N-type Ca channel	+ pr
	5-HT	-
	α-adrenergics	-
	GABA A	-
	NO	- pr
	NK_1	+ po
	AMPA	+
	A_1 adenosine	-
PAG/PMC sites	NMDA	+
	D_2 dopamine	+
	GABA A	-
	muscarinic	-
	D_1 dopamine	-
Sacral parasympathetic nucleus	AMPA, NMDA	+ po
	α1 adrenergic	+
	5-HT	-
	GABA A	- po
	Glycine	- po
Parasympathetic ganglia	VIP	+ po
	GABA A	-
	α adrenergic	-
	$5\text{-}HT_{1A}$	-
Bladder smooth muscle neuroeffector junction	M_1 muscarinic	+
	α1 adrenergic	+ pr
	$5\text{-}HT_4$	+ pr
	M_2 muscarinic	- pr
	botulinum toxin	- pr
	M_2/M_3 muscarinic	+ sm
	P_2X_1	+ sm
	NK_2	+ sm
	L type Ca channel	+ sm

+ excitatory, - inhibitory, pe = peripheral, pr = presynaptic, po= postsynaptic, sm = smooth muscle

2.2.6. The role of the Urothelium

Traditionally, the role of the urothelium was thought to be a solid barrier to prevent urine and blood contact. Recently urothelial mechanism has been described to be involved in regulating bladder function. It has been shown that urothelial cells express various sensor molecules including bradykinin, neurotrophins, purines, noradrenaline, Ach, epithelial Na channels and a number of transient receptor potential (TRP) channels. Such sensor molecules response to mechanical as well as chemical stimuli and in turn release chemicals such as ATP, prostaglandins, NGF, ACh and NO. These agents are known to have excitatory and inhibitory actions on afferent neurons, which are located close or in the urothelium. Release of ATP and NO has been detected following the urothelial strech. ATP should be acting on the P_2X_3 receptors on the sub-urothelial nerves initiating the bladder voiding (Ferguson et al, 1997). Different studies have confirmed that fact (Cockayne *et al.*, 2000; Pandita *et al.*, 2002, Ford *et al.*, 2006).

Good evidences show that there is an afferent innervation of the epithelium which involves not only afferent endings underneath the lamina propia but also afferent endings within the urothelium itself. These afferent axons have been localised in the base of the epithelium, inside the epithelium, the blood vessels and in the muscle bundles. In the mucosa all afferents axons lay either inside the epithelium or in the subepithelial plexus very close to the basal surface of the epithelium.

It has been shown that the removal of the epithelium significantly increases the contractile response of the cat bladder to electrical stimulation, potassium or betanechol (Levin *et al.*, 1995). It seems that the mucosa has a significant inhibitory effect on the contractile response of the bladder to stimulation. There are a series of mediators, including nitric oxide and ATP that may be released by the mucosa and act in the urothelium, in the afferent and efferent nerves, and on the smooth muscle itself. A summary of the possible relationships between the urothelium cells, receptors, chemical mediators and afferent neurons in the bladder can be seen in Figure 5.

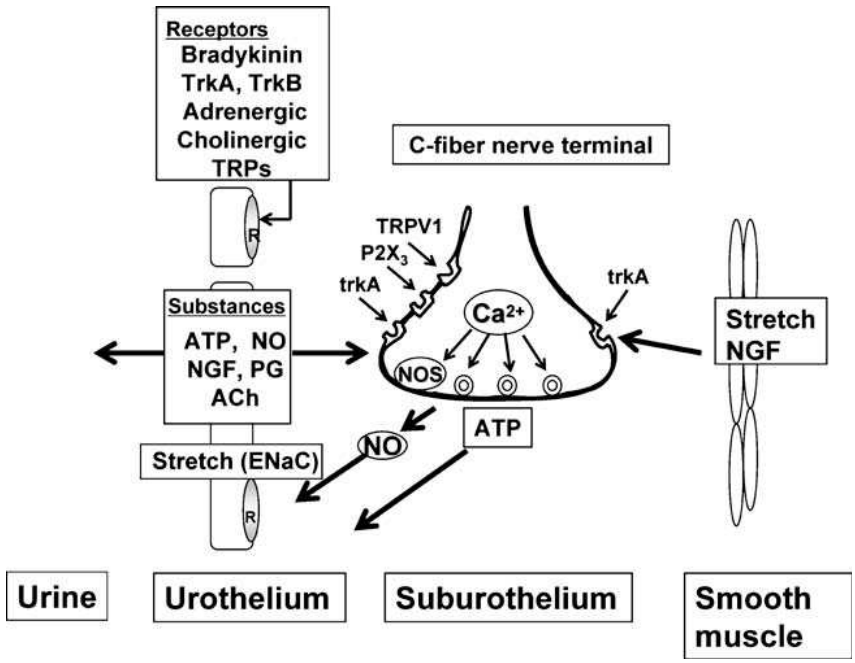


Figure 5. Interactions among receptors, chemical mediators released from urothelium and afferent nerve endings in the bladder. Various receptors are expressed on urothelial cells (bradikynin, tkrA, tkr B, adrenergic, cholinergic and TRP). ATP,NO ACh, NGF and PG can be released from urotelium via an activation of urothelially expressed ligand receptors and /or mechanoceptive receptors such as epithelial sodium channel which respond to strech of the mucosa during bladder distension. Substances released from urothelium can then stimulated receptors expressed in Cfiber afferents (P_2X_2, $TRPV_1$, tkr A).

3. MICTURITION-STORAGE REFLEX COORDINATION. PHARMACOLOGY

To permit that storage-voiding phase take place it is necessary a precise coordination of the medullar reflexes. This coordination is regulated by the PMC, avoiding the medullar nucleus to act independently. The urine storage is due to the adrenergic system. The urine voiding is due to the cholinergic system. The adrenergic system acts tonically and the cholinergic system acts under the PMC orders. During the storage of urine, distension of the bladder produces low-level vesical afferent firing, which in turn stimulated outflow to the bladder outlet and the pudendal outflow to the external urethral sphincter. These responses occur by spinal reflex pathways and represent guarding reflexes, which promote continence. Sympathetic firing also inhibits detrusor muscle and modulates transmission in bladder ganglia. A region in the rostral pons increases external urethral sphincter activity. During elimination of urine, intense bladder afferent firing activates spinobulbospinal reflex pathways passing through the PMC, which stimulate the parasympathetic outflow to the bladder and urethral smooth muscle and inhibit the sympathetic and pudendal outflow to the urethral outlet. Ascending afferent input from the spinal cord may pass through relay neurones in the PAG before reaching the PMG. Figure 6 summarised the process:

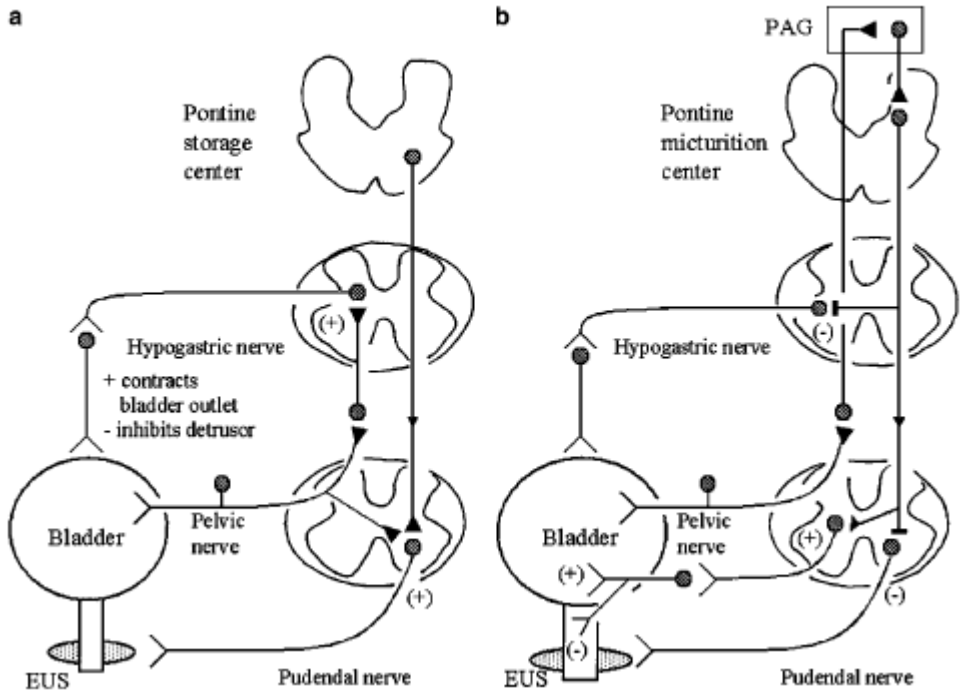


Figure 6. Summary diagram showings the neural circuits controlling continente and micturition. (a) Urine storage reflexes. (b) Voiding reflexes.

During the storage phase, the intravesical pressure is low due to the elasticity of the bladder wall. Meanwhile the bladder keeps storing urine, the neural efferent activity starts increasing. The stretch receptors in the bladder wall start sending a signal through the pelvic nerves as far as the ganglion that come back as a reflex by the same way using parasympathetic fibres. These activated fibers release Ach and contract the muscle. When the bladder is only partially full, these micturition contractions (from the release of Ach) are relaxed spontaneously, the detrusor muscle stops contracting and the pressure lows down again. This cycle is repeated and the contractions are increasing as far as the pressure gets to the needed threshold to send the sign to the PMC. The PMC informs the cortex about the bladder fullness and the need of micturition. The voiding is inhibited until it is socially possible to perform it.

The voiding voluntary process is initiated with a decrease of the urethral pressure as a voluntary relaxation of the external sphincter. The pelvic muscles also relax and the bladder neck forms a funnel. The parasymtahetic and sympathetic stimulation take place (see Figure 6) and a unique coordinated detrusor muscle contraction is produced. The high bladder pressure is the trigger of voiding.

3.1. Cholinergic system: Muscarinic receptors on bladder activation

Molecular cloning studies have revealed five distinct genes for muscarinic Ach receptors in rats and humans, and it is now accepted that five receptors subtypes correspond to these gene products (Eglen *et al.*, 1996, Caulfield, 1993). Muscarinic receptors are coupled to G- proteins, but M_1, M_3 and M_5 preferentially couple to phosphoinositide hydrolysis leading to mobilization of intracellular calcium, whereas activation of muscarinic M_2 and M_4 receptors inhibits adenyl cyclase activity (see Figure 7). Is has been suggested that M_2 muscarinic receptors stimulation may also inhibit K_{ATP} channels in smooth muscle cells from urinary bladder through the activation of protein kinase C (Bonev *et al.*, 1993). Recent studies consider that M_3 signal transduction can vary. In the human detrusor was confirmed (Schneider *et al.*, 2004) that the muscarinic receptor

subtype mediating CCh –induced contraction was the M_3 but the phospholipase C inhibitor U73122 did not significantly affected the contraction despite blocking IP3 generation. They concluded that CCh-induced contraction of human urinary bladder is mediated via M_3 receptors and largely depends on Ca2+ entry through nifedipine-sensitive channels and activation of rho-kinase (Schneider *et al.*, 2004), in addition to the IP3 pathway.

Detrusor muscle contains muscarinic receptors of the M_2 and M_3 subtype in humans (Fetscher *et al.*, 2002; Tyagi *et al.*, 2006), mice (Choppin *et al.*, 2001; Choppin, 2002), rats (Longhurst *et al.*, 1995; Hegde, 1997; Longhurst *et al.*, 2000), guinea pigs (Wang *et al.*, 1995), rabbits (Tobin *et al.*, 1995; Choppin *et al.*, 1998) and monkeys (Lai *et al.*, 1998). In most smooth muscles, the muscarinic M_2 receptor subtype accounts for 70-80% of the receptor population whereas the M_3 receptor subtype comprises only 20-30% (Eglen *et al.*, 1996). In the human bladder, the occurrence of mRNA encoding the M_2 and M_3 subtype has been demonstrated (Yamagouchi *et al.*, 1996). Recently binding studies using human biopsies from normal bladder obtained from patients show that the predominant receptor population in detrusor muscle is M_2 (71%) followed by M_3 (22%)(Mansfield *et al.*, 2005). In mucosa there is a 75% of M_2 and a 25% of M_3/M_5 (Mansfield *et al.*, 2005). Moreover, they found a negative correlation between the receptor number (Bmax) with age in detrusor muscle from male patients but not for females (Mansfiled *et al.*, 2005). In quantitative competitive RT-PCR studies it was shown a age-related decrease on mRNA for muscarinic M_3 but not M_2 receptors, very significantly in males and slightly significant in females (Mansfield *et al.*, 2005). Recently more accurate experiments evaluating differences between mucosa and detrusor mRNA expresion have shown that the increase M_2 vs.M_3 mRNA expresion with age and/or with a pathological condition (idiopathic OAB bladder) is exclusively seen in mucosa (Mansfield *et al*., 2007) However, although the predominant receptor seems to be the M_2, affinity studies on isolated bladder strips have identified the M_3 receptor subtype as the predominant receptor causing contraction of the detrusor muscle (Choppin *et al.*, 1998; Sellers *et al.*, 2000; Uchiyama *et al.*, 2004, Chess-Williams *et al.*, 2001). The major density is found in the body of the bladder (Wang *et al.*, 1995) Bladders from mutant mice lacking the receptor gene for the M_3 receptor do have impaired the detrusor contractions to CCh and the male mutants also develops urinary retention (Matsui *et al.*, 2000). The M_2 receptors, coupled to the inhibition

of adenylate cyclase, indirectly influences on the contraction via inhibition of the cAMP-mediated relaxation mediated by the sympathetic system (Hedge *et al.*, 1997). Contractile mechanisms involving M_2 muscarinic receptors, such as activation of the non-specific cationic channels and inactivation of potassium channels may be operative in the bladder (Hedge *et al.*, 1999). Contrarily to the effects seen in the mutant mice for the M_3 receptor, the animals where the M_2 receptor was suppressed did contract their bladders is a similar way that the wild type mice., although there were less sensitive to CCh (Stengel *et al.*, 2000) .In certain disease state M_2 receptors may contribute to contraction of the bladder. Thus, in the denervated rat bladder, M_2 receptors or a combination of M_2 and M_3 receptors mediated contractile responses (Braverman *et al.*, 1998; 2005). However, recent results using normal and neurogenic human bladders show that M_2 receptors do not appear to contribute to direct contraction of the human detrusor in normal or disease states. On the contrary the M_3 receptor appears to be the main responsible for the contraction in any bladder disease state (Stevens *et al.* 2006; 2007).

Muscarinic receptors may also be located on the presynaptic nerve terminals and participate in the regulation of transmitter release. There are two types of presynaptic muscarinic receptors, the one with a facilitatory mechanism and the other with an inhibitory mechanism. The facilitatory receptor is of the M_1 subtype (Somogy *et al.*, 1999). The inhibitory receptors are the M_2 in rabbit [Tobin *et al.*, 1995) and rat (Somogy *et al.*, 1992), and the M_4 in guinea pig (Alberts *et al.*, 1995) and human (D'Agostino *et al.*, 2000). See Figure 8.

Some specific muscarinic receptor functions may be changed in different urological disorders, such as outflow obstruction, neurogenic bladders, OAB without overt neurogenic cause and diabetes. However, it is not always clear what such changes mean in terms of changes in detrusor function.

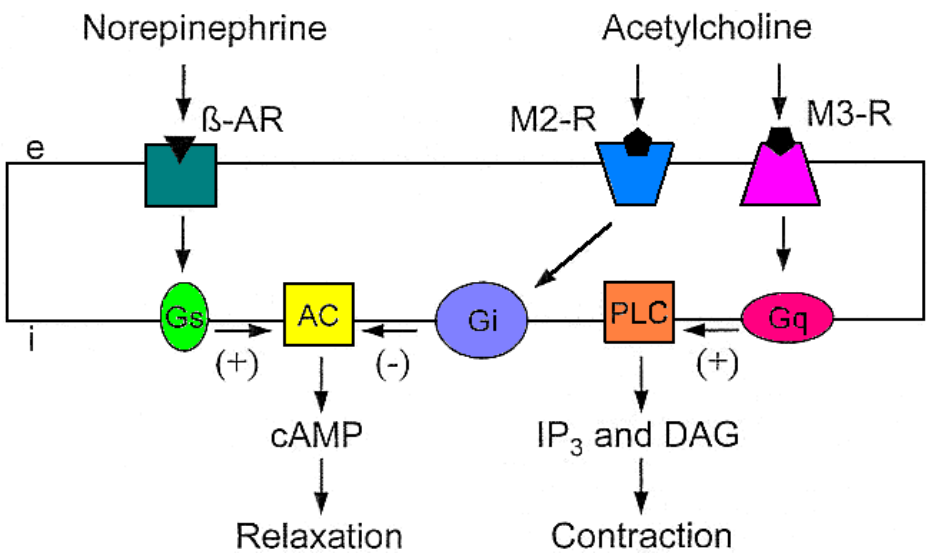


Figure 7. Interactions between β-adrenergic and cholinergic receptors on detrusor muscle stimulation.

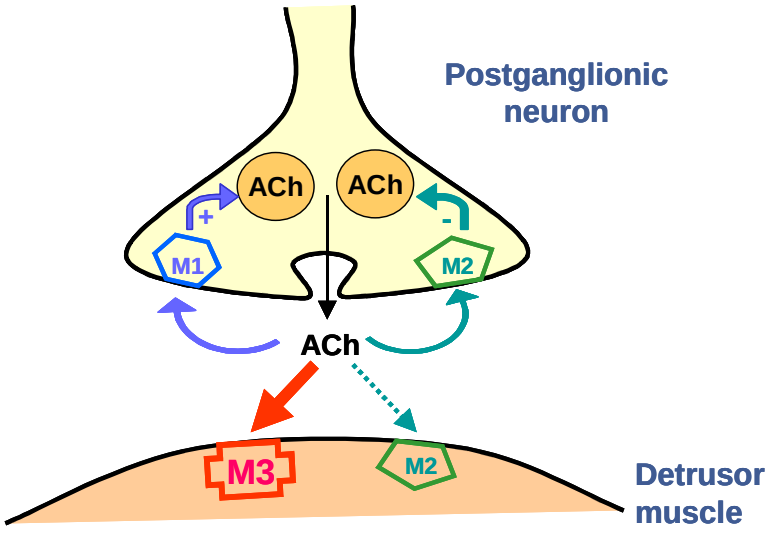


Figure 8. M_1, M_2, M_3 and M_4 receptor localization on the synapsis

Emerging evidences suggests that the urothelium contains muscarinic receptors (Abrams *et al.*, 2006; Mansfield *et al.*, 2007, Ikeda *et al.*, 2008).These receptors have been shown to release a diffusible mediator that inhibits contraction of the underlying detrusor muscle (Fovaeus *et al.*, 1999; Hawthorn *et al.*, 2000; Ikeda *et*

al., 2008). Molecular reverse transcription (RT)-PCR studies have shown the expression of mRNA for M_2 and M_3 receptors in detrusor and mucosa from control patients as well as in detrusor and mucosa of patients with refractory idiopathic detrusor overactivity. The studies showed a significant less expression of M_3 receptor mRNA in the mucosa but not in the detrusor of overactive bladder (Mansfield *et al.* 2007). Although the precise role of muscarinic receptors in the bladder mucosa it is not clear yet, it has been suggested that their stimulation could release an unknown urothelial-derived inhibitory factor (UDIF) able to modulate the detrusor contractile activity (Hawthorn *et al.*. 2000, Scott et al. 2005, Mansfield *et al.,*2007, Murakami S *et al.* 2007). Urothelial muscarinic M_2 receptors could mediate UDIF release (Scott *et al.,* 2005). It can be thought that this inhibitory factor could be decreased in the overactive bladder, causing at least in part, an increase in bladder contraction.

The specific role of each muscarinic receptor subtype in the urological tract has been pharmacologically dissected using a range of muscarinic agonists and antagonists. To help in the understanding of the experimental work described in this document, we presented the reported affinity for human muscarinic receptors subtypes of the antimuscarinics currently used for the clinical treatment of OAB (see Table 4).

Table 4. Binding affinity estimates (Ki; nM) at human recombinant muscarinic receptors (Hedge *et al.*, 2006; Ohtake *et al.*, 2007).

MOLECULE	**M_1**	**M_2**	**M_3**	**M_4**	**M_5**
Oxybutynin	1.0	6.7	0.67	2.0	11
Tolterodine	3.0	3.8	3.4	5.0	3.4
Darifenacin	7.3	46	0.79	46	9.6
Solifenacin	26	170	12	110	31
Trospium	0.75	0.65	0.5	1.0	2.3

3.2. Adrenergic system: adrenergic receptors on bladder activation

The α and β adrenergic receptors distribution in the bladder has a relevant functional role. The bladder body has an elevated concentration of β adrenergic receptors and a low concentration of α adrenergic receptors. Their activation produces the relaxation of the detrusor muscle and the storing process. Radioligand binding studies have indicated that the human detrusor possesses a population of β3 adrenceptors (Igawa *et al.*, 1999). A β3 agonist relaxes the muscle and this response is present in normal and neurogenic detrusor (Igawa *et al.*, 2001). Oppositely, the bladder neck and the urethra have a high density of post-synaptic α adrenergic receptors and their stimulation induce contraction. The subtype involved seems to be the α1. α1 antagonists are used in the treatment of benign prostatic hyperplasia thus relaxing the urethra and relieving the bladder outlet obstruction (Kumar *et al.*, 2000). This pathology can produce symptoms of OAB in severe cases. Because of that, some studies have been carried out combining an antimuscarinic drug as tolterodine with an α1 antagonist (Robinson *et al.*, 2007). The results are not conclusive enough and further trials are needed to establish the advantage of this combination.

Thus, adrenoceptors in LUTs predominantly mediate continence-supporting functions. Adrenergic agonist have been used in the treatment of stress incontinence (Jonas, 1977). Attempts have been made to develop agonists with selectivity for the human urethra. α1A seems to predominate in the human lower urinary tract (Andersson *et al.*, 2001). However, the receptor with low affinity for prazosin (α1L), which has not been cloned and represents a functional phenotype of the α1A receptor, was found to be prominent in the human urethra (Canda, 2008). Unfortunately, the lack of drugs with an appropriated sub-type selectivity make difficult to establish the role of the α-agonists in the treatment of UI.

3.3. Other relevant systems

As described in 2.5.5., other potential targets can be explored to improve the OAB. Up to date, only the following mechanisms, both central and peripheral, have been studied up to clinical trials (Table 5). The ones described in Table 6

are in clinical trials at the moment of writing this work (Table 6). Thus, only muscarinic receptors remain the unique established target.

Table 5. Current status of mechanisms of action different from muscarinic

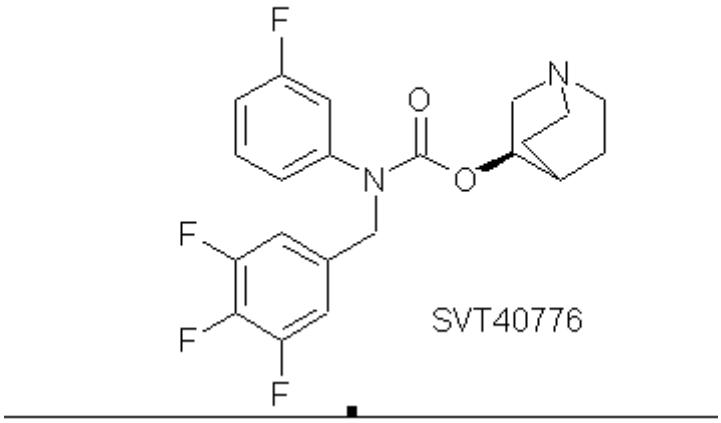
MECHANISM	STATUS
Ca2+ channel blockers and K+channel openers	failed because of the lack of selectivity.
Vanilloid receptors	failed to demonstrate efficacy in humans (Proof of concept (POC)).
5HT1A	failed to demonstrate the POC
Tachykinin NK-1 antagonists	currently under phase II. Published data on ICS 2006: Aprepitant showed lower efficacy than antimuscarinics. No more data in 2007.
β3 agonists	phase II completed. Not very good results in efficacy. Taquicardia.

Table 6. Mechanisms of action different from muscarinic under current clinical investigation

MOLECULE	MECHANISM	COMPANY	STATUS
Solabegron	Beta-3 adrenoreceptor agonist	GSK	Phase II
YM-178	Beta-3 adrenoreceptor agonist	Astellas	Phase II
Aprepitant	Tachykinin NK-1 antagonist	Merck	Phase II
Casopitant	Tachykinin NK-1 antagonist	GSK	Phase II
SSR-240600	Tachykinin NK-1 antagonist	Sanofi-Aventis	Phase II

4. SVT-40776: CHEMICAL AND PHYSICAL DESCRIPTION

Chemically the compound is a quinuclidyl *N*-phenyl-*N*-alkyl carbamate belonging to a family of compounds of general formula (I) acting as a selective M_3 muscarinic receptor antagonist (Farrerons *et al.*, 2002).



A chiral synthetic process allows preparing the compound as a pure enantiomer. A non-hygroscopic pharmaceutically acceptable salt has been obtained, what makes the product easier to handle and to store.

The free base has a molecular weight under 450.

Batches: Biological results described in this document were obtained with a range of lab-scale batches (e.g. L-2, L-3, L-4…L-12) with a minimum purity of 94% and the first kg-scale batch, a cGMP fulfilling, was obtained in January 2003 and named D-1 with a purity higher than 99.7%. This batch was also used for the preclinical development of the compound up to Phase I clinical trials.

OBJECTIVE

The main objective of this work was to characterise the pharmacology of SVT-40776 as a potential drug directed to treat OAB. This compound lacks any relevant affinity for human M_2 receptors, presenting a 100-fold selectivity ratio M_3 *vs.* M_2. We postulated that a M_2 sparing muscarinic antagonist would be predictive of a safe cardiovascular profile. In addition, we wanted to prove that M_2 antagonism is not a requirement to achieve the effectiveness of the classical non-selective muscarinic antagonists inhibiting the cholinergic bladder contractions observed on the OAB patients.

The specific goals of this work are detailed as follow:

1. To characterize the binding affinity and selectivity of SVT-40776 in the muscarinic receptor family. Thus, to evaluate the affinity constant (Ki) of the compound on binding in CHO cell membranes expressing separately the five human muscarinic receptor subtypes. To determine the binding characteristics to the M_3 receptor. To evaluate the nature of the binding of SVT-40776 to the M_3 receptor using competition-binding protocols.

2. To determine its functional activity in bladder and heart. Thus, to evaluate the functional antagonism of the compound on cholinergic bladder contraction in mouse, guinea pig and dog, associated with OAB contractions and compare it to the activity obtained in the atria contraction, associated with cardiac effects.

3. To determine its *in vivo* fuctional profile on the urodynamia of anaesthetised guinea pig.

4. To determine the time course of its therapeutic effect in mice bladder. Thus, to evaluate the duration of action on bladder muscle contraction after oral administration in order to integrate its pharmacokinetic and distribution pattern.

MATERIAL AND METHODS

1. MATERIAL

1.1. Animals

Male CD-1 mice (25-30 g) and female Hartley guinea pig (300-350 g) were obtained from Harlan Iberica (Sant Feliu Codines, Sant Miquel Fai, km 3- Spain). Female Hartley guinea pigs (350-450 g) were obtained from Charles River-France. Animals were housed in groups (7 mice/cage; 6 guinea pig/cage) with free access to tap water and commercial pelled food (Mice: Teklad 2014, Harlan, Italy; Guinea Pig: Teklad 2040) and kept at 20 (±1ºC) at a humidity of 60% (±10%) under a 12h light-dark cycles (lights on at 7:00 a.m.) for a week before use. Animals with no abnormalities in their general signs and behaviour were selected for the experiments. In all protocols animals were divided at random into the treatment groups before the start of each experiment.

Dogs were obtained from Isoquimen (Sant Feliu Codines, Sant Miquel Fai, km 3-Spain). Animals were housed in groups of 2 dogs per kennel with free access to tap water and commercial pelled food (Teklad 2021) and kept at temperatures 18 ± 2ºC at a humidity of 60% (±10%). Animals with no abnormalities in their general signs and behaviour were selected for the experiments and were recycled. The animals were used up to a maximum of 4 times with one month-washout period between operations.

All animal procedure was conducted according to the Real Decret 1989/Generalitat guidelines following the SALVAT Standard Operative Procedures (SOPs) approved by SALVAT Quality Unit Assurance. Each experimental protocol was approved by SALVAT Laboratories Experimental Animal Ethical Committee (CEEA) and the Isoquimen CEEA (for those experiments carried out on its facilities) and subsequently approved by the national organism responsible for it (*Dpt. Remadaria i Pesca and Dpt. Medi Ambient; Generalitat de Catalunya, Spain*).

1.2. Products

SVT-40776, darifenacin, solifenacin and tolterodine were synthesized by Medicinal Chemistry Dpt (SALVAT). Atropine, acetylcholine (ACh), 4-DAMP, 4-DAMP Mustard, methoctramine, pirenzepine, oxybutynin, indomethacin, hexamethonium, pilocarpine and CCh were obtained from Sigma Chemical Co, (St Louis, U.S.). 4-DAMP mustard was purchased from Research Biochemicals International (Natick, MA, USA) Radiolabeled compounds, so [^{3}H]-N-methyl scopolamine ([^{3}H]NMS) (79-84 Ci/mmol) was obtained from Amersham Biosciences.

For binding studies SVT-40776, darifenacin, solifenacin, tolterodine, 4-DAMP, 4-DAMP mustard, methoctramine and oxybutynin were dissolved in 100% DMSO to prepare a concentrated stock solution of 10 mM. Chinese hamster ovary (CHO-K1) cell membranes containing human muscarinic receptors (M_1-M_5) were obtained from PerkinElmer (MA, USA). Phosphate buffered saline (PBS) was prepared in-house (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4).

For isolated tissue experiments SVT-40776, darifenacin, solifenacin and tolterodine, 4-DAMP, 4-DAMP mustard, oxybutynin, methoctramine and pirenzepine stock solutions were prepared at 1 mM in DMSO and dilutions made in distilled water. For indomethacin, hexamethonium and CCh s*tock* solutions were prepared at 5mM (CCh) or 10 mM (others) on distilled water. Krebs buffered solution was prepared in-house (see 2.3.).

For mice *ex vivo* experiments SVT-40776, tolterodine, darifenacin, oxybutynin and solifenacin were freshly suspended in vehicle (HPMC 0.5 % and Tween 80® 0.1 %) 1h before oral administration (10 mL/kg). For indomethacin, hexamethonium and CCh s*tock* solutions were prepared at 5mM (CCh) or 10 mM (others) on distilled water. Krebs buffered solution was prepared in-house (see 2.3.).

For dog *ex vivo* experiments SVT-40776 and solifenacin were freshly dissolved in vehicle (HPMC 0.5 % and Tween 80® 0.1 %) 1h before oral administration (2 mL/kg). For indomethacin, hexamethonium and CCh s*tock* solutions were prepared at 5 mM (CCh) or 10 mM (others) on distilled water. Krebs buffered

solution was prepared in-house (see 2.3.). Enrofloxacin, meloxicam, acepromazine and buprenorphine were obtained from the local veterinarian products distributor.

For functional *in vivo* anaesthetised animal experiments stock solutions of SVT-40776 and solifenacin were diluted in saline. Tolterodine were diluted in distilled water. Darifenacin was diluted in 10% DMSO in distilled water.

2. METHODS

2.1. Binding and cell functional studies

2.1.1. Human muscarinic receptor binding affinity and selectivity of SVT-40776

In order to carry out these experiments, selective and non selective antagonists have been used. The affinity of these pharmacological tools to the five human muscarinic receptor subtypes had been characterized using radioligand studies (Hedge, 1997, 2006; Eglen, 2001; Ikeda, 2002;; Ohtake, 2007) (see Table 4. in Introduction 3. Micturiton-Storage reflex coordination: pharmacology).

These studies consist on evaluating the competition profile of the compound (I) with an unknown affinity in front of a known radioactive compound (A) with a high and established affinity for the receptor R. The capacity of displacement of the compound I to the binding of A is an indicator of the affinity of I for the receptor R.

The concentration of I enough to induce a 50 % displacement is the $IC_{50.}$ The inhibition constant *Ki* ($Ki=IC_{50}/1+[A]/K_{D,}$ K_D= A dissociation constant) indicates the I affinity for the receptor.

2.1.2. Membrane preparation

Membranes containing muscarinic receptors were obtained from two different sources:

1. M_1, M_2 and M_3 containing membranes were prepared from CHO-K1 cells stably transfected with the human receptors obtained from the National Institute of Health. Each cell line was grown to 80% confluency. Trypsinized-harvested cells were washed and homogenized in ice-cold PBS and the resulting membranes were pelleted by centrifugation at 30,000 g for 30 min. Protein content was determined using the Bio-Rad protein assay system, and membranes were stored in aliquots at -80ºC before use.

2. In order to establish the affinity and selectivity *versus* all muscarinic receptors (M_1-M_5), CHO membranes containing one single muscarinic receptor were obtained from Receptor Biology Inc.

2.1.3. Saturation binding studies with [^{3}H]NMS

Saturation curves were performed incubating different concentrations of [^{3}H]NMS with 25 µg of the membrane preparation and increasing concentrations of antagonist in PBS. Incubation was carried out in a total volume of 200 µL at 25ºC for 1 h. Membrane homogenates were preincubated for 1.5 h with the antagonist before adding the radioligand. Non-specific binding was defined in the presence of 5 µM atropine. The binding reaction was terminated by filtration over presoaked (0.5% PEI) 96-well glass filter plates (Millipore; type FC) using a Multiscreen vacuum manifold (Millipore). The filters were washed 3 times with ice-cold 50 mM Tris-HCl, 0.9% NaCl (pH 7.4), dried and 10 µL of Betaplate scintillation liquid (PerkinElmer) were added to each well. The retained radioactivity was quantified in a liquid scintillation counter (Microbeta® TriLux; PerkinElmer).

2.1.4. Competition studies with [^{3}H]NMS

Competition curves were determined incubating 0.5 nM [^{3}H]NMS with 20-25 µg of the membrane preparation and different concentrations of antagonist in a total volume of 200 µL for 1 h at 25ºC. Non-specific binding

was defined in the presence of 5 µM atropine. The reaction was stopped and radioactivity quantified as described above.

Data analysis

Data points derived from the specific binding were analyzed by non-linear curve-fitting using Prism 4.0 (GraphPad Software®, San Diego, CA, USA). Binding parameters were obtained as the best-fit values for the data using the least-squares method. Data was compared using paired Student's *t*-test.

2.2. Isolated tissue functional studies

2.2.1. Tissue preparation

The animals were sacrificed by decapitation and their urinary bladders were removed. The way of sacrifice was accepted by the regulatory animal guidelines (FELASA) due to any other system using a pharmacological tool could have an effect over the organs under study, Thus, it is recommended to use a mechanical procedure in those protocols were the histology, biochemistry or functionally of the organ or tissue has to be studied. Once the animal was opened through its longitudinal abdominal line, the bladder was easily localised on the abdominal inferior cavity (Fig 2.3.a). The bladder was carefully dissected taking care in not scratching the walls. It was then put down in a Petri disk containing Krebs solution with the dorsal face on the plate. The identification of the bladder ventral and dorsal faces is crucial to obtain afterwards very accurate results. The dissection procedure was exactly the same for all bladders in order to obtain the most identical strips between animal and minimize the inter-animal variation. Once the bladder was facing its ventral face to the researcher, a vertical cut was made in order to open the bladder (see

Figure 9). The bladder was then extended and fixed with a dissection needles. Two parallel blood vessels could be observed crossing longitudinally the bladder body. The tissue between these two vessels was then dissected in strips of approximately 4 mm long x 2 mm wide.

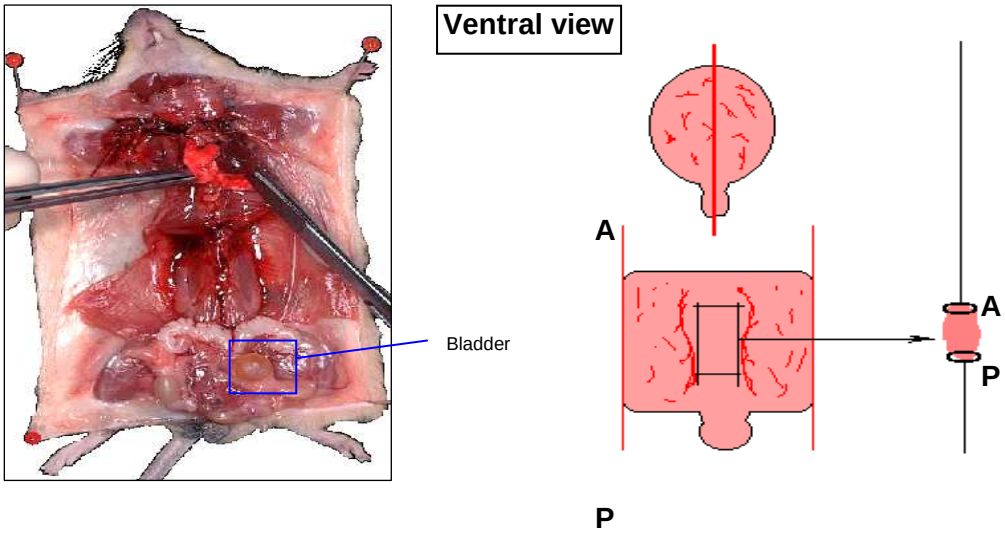


Figure 9. Animal dissection in order to obtain detrusor strips.

For the *ex vivo* experiments tissues were prepared using the same procedure. The difference was that the animals were previously administered by oral route 3 h before the extraction of the tissues.

The strips were then suspended in a 25 mL organ bath (Automated 4 chambers Panlab Organ baths) containing Krebs solution at pH 7.4 (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.6, KH2PO4 1.5, CaCl 1.5 and glucose 11) Krebs solution was continuously perfused by a gas mixture of 95% oxygen and 5% carbon dioxide, and kept in a constant 37ºC temperature by a circulation bath. The strips were anchored to the base of the bath using a suture silk to the holder (see Figure 10). The other end was suspended by a silk suture to a pure force isometric transducer (Cibertec). The force produced for these strips was measured for these transducers and recorded using the specific software *PowerLab* (*ADInstruments*; Australia).

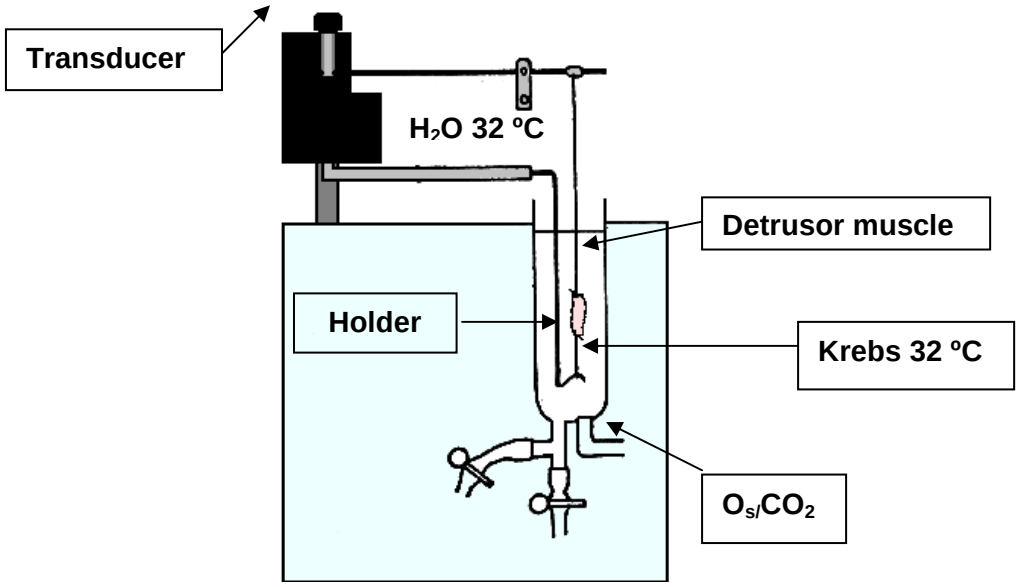


Figure 10. Organ bath. Detail of one of the chambers

2.2.2. Effect of SVT-40776 on mice isolated bladder *in vitro*

Once the strips were attached to the transducers, a period of stabilization was needed to warm up the tissues. The resting tension was 0,5 g and during one hour (stabilization period) this tension was readjusted every 10 min. The tissues tend to relax thus this stabilization period has the objective of relaxing the strip to its maximum in order to have all tissues with the same cell distension. In addition, the tissue was washed every 15 min, so the Krebs solution was continuously replaced. The viability of each tissue was assessed by determining the contractile response to KCl (120 mM) at the start of the experimental protocol. After washing, tissues were reequilibrated for 15 min and allowed to regain baseline tension. When the KCl challenge did not produce a contraction or the contraction was considered too small the strip was discarded.

Next step was to warm up the tissues with repetitive contractions of 3 µM of CCh (a non-selective muscarinic agonist) (Figure 11). This is done in order to obtain the maximal sensitivity and stability. CCh was in contact with the strip for one and half minute and then washed. A good warming of

the tissue is established when a minimum of three comparable contractions (variation between contractions less than 5%) are obtained.

Thus, now the tissue was ready to start the the evaluation of the pharmacological response of the detrusor. Cumulative consecutive concentration-effect curves to CCh were then constructed in each bladder preparation. Antagonist was incubated for a 60 min period between curves.

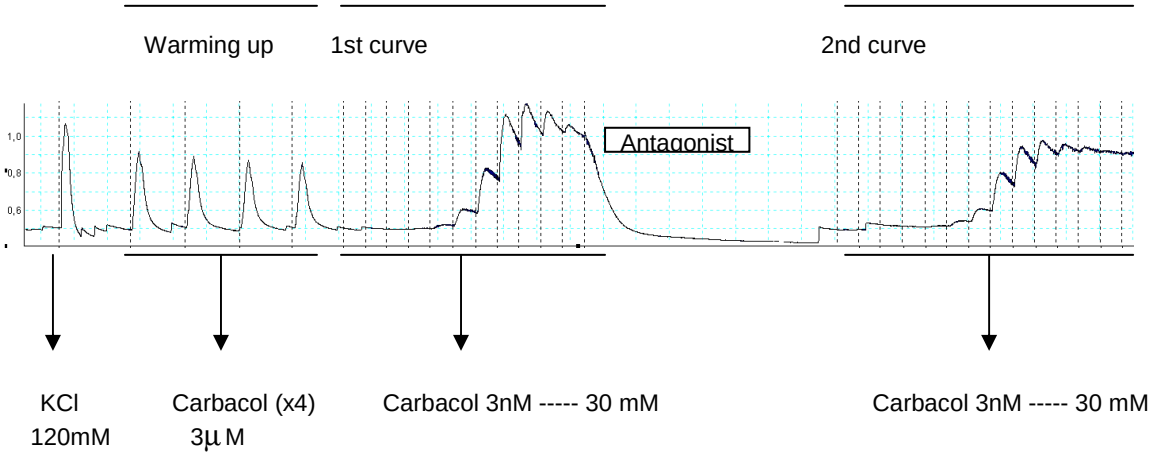


Figure 11. *In vitro* experimental protocol

The cumulative curve was constructed using increasing CCh logaritmic concentrations (3nM, 10nM, 30nM, 100nM, etc). This protocol permits calculate accurately the EC_{50} for agonists and the pA_2 for antagonists. Both parameters are extensively used to characterise agonists and antagonists. EC_{50} is the concentration of the agonist necessary to produce a 50% of its maximal response. pA_2 is the concentration of the antagonist necessary to produce a 2-fold displacement of the EC_{50}.

The two curves used in this protocol started with CCh 3nM and finished at 30 mM (3nM, 10nM, 30nM....3µM,10µM....to 30 mM). The tissue was incubated with each one of these concentrations for five minutes. After this period, the adequate volume was administered to obtain the next concentration in the bath.

Once we obtained the first curve (up to 30mM) the preparation was washed and permitted the tissues to equilibrate. Once the resting tension was obtained, the antagonist was dosed into the bath and left for one hour. Then, a second cumulative curve was constructed.

Indomethacin and hexamethonium were used to decrease spontaneous contractions due to endogenous prostaglandins and putative nicotinic receptors playing a role in the bladder contraction respectivelty.

2.2.3. Effect of SVT-40776 on mice isolated bladder *ex vivo*

Groups of mice (n=4-6/dose) received a single oral dose (0.3 to 50 mg/kg, 10 ml/kg) of vehicle, SVT-40776, tolterodine, darifenacin and solifenacin (3-5 doses/compound). Mice were sacrificed 1h, 3h, 5h and 24h later and urinary bladder was excised and prepared as described before and placed in 25 ml organ baths containing Krebs solution maintained at 37ºC and aerated with 95%O_2/5%CO_2. As mentioned before, the Krebs solution for detrusor routinely contained indomethacin (30 μM) and hexamethonium (0.1 μM). Pure isometric transducers were used for all experiments. Contractile force in isolated longitudinal strips of bladder detrusor muscle and beating frequency on isolated spontaneously beating atria were measured. After tissue equilibration, the viability of each strip was assessed by determining the contractile response to KCl (90 mM) at the start of the experimental protocol. Only repeated KCl was used in this case to warm up the tissue. A unique cumulative concentration-effect curve to CCh was then constructed in each tissue and referred to the 90 mM KCl effect.

2.2.4. Effect of SVT-40776 on dog isolated detrusor muscle *ex vivo*

Fasted male dogs (n=2/dose/time) received a single oral dose (1-10 mg/kg) of vehicle or SVT-40776. 1h or 3h later a bladder biopsy was obtained. Dogs were treated with enrofoxacin (5 mg/kg), meloxicam (0.2 mg/kg), acepromazine (0.1 mg/kg) and buprenorphine (0.01 mg/kg) i.m. 30 min before the surgical procedure. The anaesthesia was induced with thiopental iv (1:20; 6-10 mg/kg) and maintained with intratraqueal mixture of isofluorane (1.5%) and oxygen (2%). The duration of the procedure was about 20 min. Once the animal loses the reflexes a lateral longitudinal caudal incision was made up to acces the bladder that was emptied through urethral cannulation. An incision on the serosa and detrusor was made from the dorsal face and a piece of 0.7 cm x 0.4 cm of detrusor was dissected. The bladder was then sutured without perforing the mucosa layer. The animal was let to recover and reused 4 weeks later. Each animal provided between 3-4 samples. As said, mucosa layer was left in the bladder and serosa was separated after the dissection. Once the muscle was clean from serosa, strips of tissue (8x3mm) were prepared. Tissues were placed in 25 mL organ baths containing Krebs solution maintained at 37ºC and aerated with 95%O_2/5%CO_2. The Krebs solution for detrusor routinely contained indomethacin (30 µM) and hexamethonium (0.1 µM). Pure isometric transducers were used for all experiments. Contractile force in isolated longitudinal strips (2x1 mm) of bladder detrusor muscle was measured. The resting tension was 1g and during one hour (stabilization period) this tension was readjusted every 10 min. Afterwards we followed the same protocol as described in 2.2.3.

2.2.5. Data Analysis

In vitro experiments

Bladder contractions were registered as changes in the baseline tension and were expressed as a percentage of the maximum response (Emax) of CCh in the first cumulative curve. The cumulative curves were analysed by non-linear curve-fitting using Prism 4.0 (GraphPad Software®, San

Diego, CA, USA), where the values of pEC_{50} (pEC_{50}= -log EC_{50}) were calculated for each curve. Concentration-ratio or dose-ratio (dr) was determined from pEC50 values in the presence and absence of different antagonist concentrations. pA2 values were obtained from the x-axis intercept from the Schild-plot method (Arunlakshana & Schild, 1959) and given as means with 95% confidence interval. When maximum responses were significantly affected by the antagonist, the x-axis intercept was interpreted as an apparent pA_2. All other data are shown as mean ± s.e.mean of 4-8 experiments.

Ex vivo experiments

CCh concentration-response curves were fitted as described before in the *in vitro* protocol. Concentration-ratio (dr) was determined from pEC50 values considering curves obtained from vehicle-treated groups as 'control pEC50' and curves from antagonist-treated animals as 'treated animals pEC50'. A pA2-equivalent dose (pA2-ED) value was obtained from the Schild-plot method, using the oral doses instead of bath concentrations for each compound and given as means with 95% confidence interval. When maximum responses were significantly affected by the antagonist, the x-axis intercept was interpreted as an apparent pA_2-ED. All other data are shown as mean ± s.e.mean of 4-6 experiments.

Calculation of pA_2

The pA_2 is a measure of the potency of antagonists, mainly when they behave as reversible competitive antagonists. Where A is the agonist, and B is the antagonist, the competitive binding is when B is using the same receptor than A and the cumulative dose-response curve for A, constructed in presence of B, is displaced paralleled to the right (Figure 12).

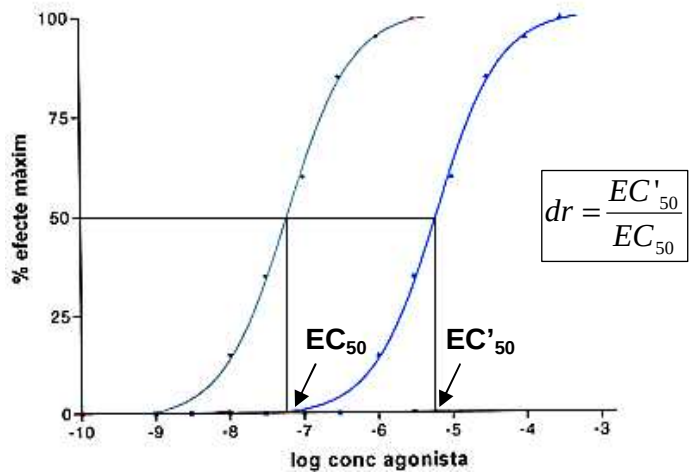


Figure 12. Cumulative dose-response curve for a competitive reversible antagonist.

The receptor occupancy due to A is *f* (

Figure **13**).

$$f = \frac{[A]}{[A] + K_a\left(1 + {[B]}/{K_b}\right)}$$

Figure 13. Receptor occupancy (*f*). [A]= A concentration, Ka= A dissociation constant A; [B]= B concentration, Kb= B dissociation constant

The antagonist increases the EC_{50} with a factor of $1+[B]/K_b$. This factor is the dose-ratio (dr). If the two curves are parallel, the dr can be calculated in any point, but to assure a more accurate calculation the best point to use is the EC_{50}. Thus, the dr is calculated as the EC_{50} in the presence of the antagonist (EC'_{50}) divided for the EC_{50} in the absence of the antagonist. As a result, $dr = EC'_{50}/ EC_{50}$ and from here it is deduced that:

$$\frac{\mathrm{EC'_{50}}}{\mathrm{EC_{50}}} = 1 + \frac{[B]}{K_b} \qquad \frac{\mathrm{EC'_{50}}}{\mathrm{EC_{50}}} - 1 = \frac{[B]}{K_b} \qquad dr - 1 = \frac{[B]}{K_b}$$

When the equation is represented in logarithmic:

$$\log(dr - 1) = \log[B] - \log K_b$$

$$pA_2 = -\log K_b$$

Thus, a graph of the log*(dr-1) vs.* log*[B]*, called *Schild's plot* (Figure 14), will represents a straight line with a slope around the unit (between 0.8 and 1.2) that will cross the abscissa axe in $logK_b$. Following the notation for pH and pK, the potency of an antagonist is expressed as the pA_2. Under a competitive antagonism conditions, the pA_2 is then the negative logarithm of the antagonist concentration (Molar) needed to obtain the dr of the agonist equal to 2 (dr = 2).

The pA_2 do not depend on the agonist used and the value obtained is an intrinsic measure of the potency of the antagonist. It only depends on the tissue used.

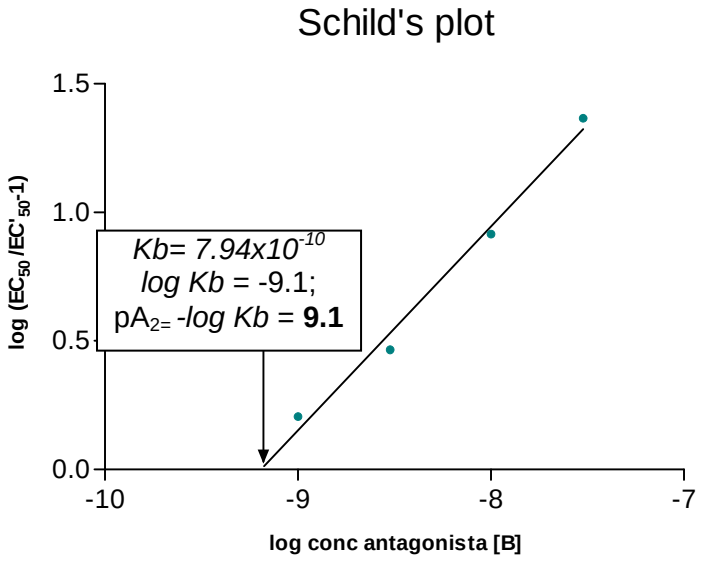


Figure 14. Schild's Plot

In addition, the Schild's Plot analysis allows the study of the type of antagonism that it has been produced. As we have mentioned before, the pA_2 measurements had been designed for competitive and reversible antagonisms. Thus, variations in the parameters obtained can suggest different kinds of antagonism.

2.3. Whole animal functional studies

2.3.1. Effect of SVT-40776 on anaesthetised guinea pig urodymania

Isovolumetric model

Guinea pigs (n = 4-6 animals/ compound) were anaesthetised by urethane 1.5 g/kg i.p.. A catheter (PE-50 polyethylene catheter) was implanted in the bladder via urethra and the bladder empted of urine. Carotid and jugular vessels were cannulated to register arterial pressure (MAP) and as administration route respectively. Bladder and carotid catheters were connected to pressure transducers (Transpac IV) and analysed using PowerLab® Software (ADI System) Software. Bladder was filled with 2.8 ml of saline to obtain a mean pressure of 58.5 (± 2.8) mmHg. Through carotid artery, a baseline MAP of 59.8 ± 1.7 mmHg was registered. After obtaining stable bladder spontaneous contractions, the compound was administered by intravenous bolus followed by a cumulative consecutive dose-response protocol (15 min between doses or when stable contractions were obtained). Responses were measured on the first 5 min or 15 min post-administration period for MAP and bladder contractions respectively.

Cystometry

Guinea pigs (n = 4-6 animals/dose) were anaesthetized with urethane 1.5 g/kg i.p. The abdomen was opened through a midline incision and a polyethylene catheter (PE-50) was implanted in the bladder through the dome and secured with silk thread for simultaneous bladder filling and recording of pressure. Carotid artery and jugular vein were cannulated to register arterial pressure (MAP) and as administration route, respectively. Bladder and carotid catheters were connected to pressure transducers (Transpac IV) and analysed using PowerLab® Software (ADInstruments). The bladder catheter was connected via a T-tube to the pressure transducer and to an infusion pump. After manually emptyng the bladder, it was allowed to equilibrate for 10 min. Then, a saline bladder infusion

was started at 0.1mL/min and a complete filling phase was recorded until micturition occurred (micturition cycle). The infusion pump was left for one additional min and stopped. The bladder was then manually emptied, left for 5 min and a new filling phase was initiated. Four reproducible phases or micturition cycles could be carried out on the same animal. The protocol consisted in a first phase to be used as baseline, followed by three more phases where intravenous saline was administered at the same time that the infusion pump was started. In the case of treated animals, the compound was administered at the beginning of the last micturition cycle. Time-maching controls were carried out. The following parameters were evaluated:

- bladder capacity (BC, mL), defined as the volume of saline infused into the bladder and necessary to induce micturition contraction.
- micturition pressure (MP, cm H_2O) as maximal intravesical pressure induced by contraction of the destrusor during micturition.
- threshold pressure (TP, cm H_2O), the pressure value recorded just before initiation of the micturition contraction.
- residual pressure (RP, cm H_2O), the resting pressure obtained after micturition.

2.3.2. Data Analysis

Isovolumetric model

Amplitude from all bladder contractions (intravesical pressure) was measured during the 15 min period between doses and an amplitude mean calculated for baseline and for each dose. The same calculation was applied for the MAP but using the first 5 min post-dosing. Percentage of variation was calculated *vs.* baseline effect. Thus, this percentage was plotted against the doses and a dose-response curve was obtained. An ED_{25} was calculated when possible. A non-paired Student's *t*-test was used for statistical analysis.

Cystometry

A delta of variation was calculated between the parameters obtained for the third saline micturituion cycle *vs*. the last micturition cycle. A dose response was carried out with the SVT-40776 and most representative competitors but ED_{50} only could be calculated for SVT-40776 and only for two parameters as no clear dose responses were found for the rest of compounds tested and/or parameters evaluated. Thus, to be able to make a correct comparison between the drugs tested, the results are only presented as deltas for each parameter calculated: BC, MP, TP and RP. . A non-paired Student's *t*-test was used for statistical analysis.

2.4. Functional selectivity studies

2.4.1. Effect of SVT-40776 on mice isolated atria *in vitro* and *ex vivo*

Animals were sacrificed by decapitation and both atria were excised and quickly placed in aerated Krebs' solution (without either indomethacin or hexamethonium). Right and left atria were very carefully ligated taking care of not breaking the atria sinus. In this case we did use the own heart beating system so electric stimulation was not required in order to induce contractions. For the *ex vivo* assays the animals were pretreated with an oral dose of the compound and sacrificed 3 h later. The tissues followed then the same procedure described before.

For *in vitro* experiments compounds were tested in the isolated tissue bath, using the same procedure that for the *in vitro* detrusor. For the *ex vivo*, groups of mice (n=4-6/dose) received a single oral dose (0.3 to 50 mg/kg, 10 ml/kg) of vehicle, SVT-40776, tolterodine, darifenacin and solifenacin (3-5 doses/compound). Mice were sacrificed 3h later and atria were excised and prepared as described before. Then, they were placed in 25 ml organ baths containing Krebs solution maintained at 37ºC and aerated with 95%O_2/5%CO_2. As mentioned before, the Krebs solution for detrusor routinely contained indomethacin (30 µM) and hexamethonium (0.1 µM). Pure isometric transducers were used for all experiments.

Contractile force in isolated longitudinal strips of bladder detrusor muscle and beating frequency on isolated spontaneously beating atria were measured. After tissue equilibration, the viability of each strip was assessed by determining the contractile response to KCl (90 mM) at the start of the experimental protocol. Only repeated KCl challenges were used in this case to warm up the tissue. A unique cumulative concentration-effect curve to CCh was then constructed in each tissue and referred to the 90 mM KCl effect.

2.4.2. Data Analysis

CCh concentration-effect curves were fitted as described before in the *in vitro* protocol, Concentration-ratio (dr) was determined from pEC50 values considering curves obtained from vehicle-treated groups as 'control pEC50' and curves from antagonist-treated animals as 'treated animals pEC50'. A pA2-equivalent dose (pA2-ED) value was obtained from the Schild-plot method, using the oral doses instead of bath concentrations for each compound and given as means with 95% confidence interval; all other data are shown as mean ± s.e.mean of 4-6 experiments.

RESULTS

1. BINDING STUDIES

1.1. SVT-40776 receptor affinity and selectivity

The affinity and selectivity of SVT-40776 for the human M_3 mACh receptor subtype was first determined by [^{3}H]NMS receptor binding competition in CHO cell membranes containing muscarinic receptors. Receptor binding affinities were also determined for a range of well-known muscarinic antagonists such as oxybutynin, tolterodine, darifenacin and solifenacin. Equilibrium dissociation constants (K_d) values for [^{3}H]NMS at M_1-M_5 were determined by saturation binding analysis, in order to calculate the affinities (K_i) of the competing compounds using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). The affinity and selectivity (expressed as K_i M_x/M_3 ratio) of the antagonists for the different mACh receptor subtypes are summarized in Table 7, Table 8 and Table 9.

Table 7. Affinity and selectivity estimates of antagonists in radioligand binding assays at M_1-M_3 recombinant human muscarinic receptor subtypes.

COMPOUND	BINDING AFFINITIES (Ki values, nM)			RATIOS	
	CHO-hM_1	CHO-hM_2	CHO-hM_3	M_2/M_3	M_1/M_3
Atropine	1.1 ± 0.9 (0.2-1)	1.3 ± 0.1 (0.5-1.2)	1.8 ± 0.04 (0.15-1.2)	0.7	0.6
4-DAMP	2.4 ± 0.5 (0.6-1.2)	10.9 ± 0.5 (4-16)	0.8 ± 0.3 (0.5-1.2)	13.7	3.1
Methoctramine	260 ± 117 (16-80)	98 ± 61 (15-16)	3321± 1573 (125-501)	0.03	0.1
Oxybutynin	3.6 ± 1.2 (2.4-3.5)	5.9 ± 3.2 (6.0-16)	2.0 ± 0.5 (0.7-2.0)	2.9	1.8
Tolterodine	4.7 ± 1.5 (3.2)	3.5 ± 0.03 (4.0)	8.6 ± 1.7 (3.4)	0.4	0.5
Darifenacin	53 ± 23 (16-41)	163 ± 25 (40-100)	2.9 ± 1.1 (1.2-4)	55.0	17.9
Solifenacin	1.0 ± 0.06 (25)	13.4 ± 2.4 (120)	1.6 ± 0.05 (10)	8.6	0.6
SVT-40776	0.7 ± 0.23	40.0 ± 16.0	0.32 ± 0.12	125.0	2.2

Footnote: Membranes were prepared from CHO cells. Values are expressed as the mean of, at least, two independent experiments, eight points per curve and each point performed in duplicate. Hill slopes of competition curves were not significantly different from unity. Numbers in parentheses indicate the values described in the literature.

Table 8. Affinity estimates of antagonists in radioligand binding assays at M_1-M_5 recombinant human muscarinic receptor subtypes.

COMPOUND	BINDING AFFINITIES (Ki values, nM)				
	hM_1	**hM_2**	**hM_3**	**hM_4**	**hM_5**
Oxybutinin	3.8 ± 0.4 (2.4-3.5)	9.2 ± 0.95 (6.0-16)	1.6 ± 0.3 (0.7-2.0)	1.7 ± 1.2 (2.0-6.3)	5.3 ± 0.65 (11-25)
Tolterodine	2.6 ± 0.53 (3.2)	2.2 ± 0.45 (4.0)	4.13 ± 1.7 (3.4)	1.8 ± 0.16 (5)	2.3 ± 1.1 (2.5)
Darifenacin	50.5 ± 3.9 (16-41)	88.7 ± 3.2 (40-100)	3.05 ± 0.2 (1.2-4)	20.9 ± 7.4 (10-20)	7.4 ± 0.62 (8-10)
Solifenacin	2.5 ± 0.5 (25)	43.2 ± 9.8 (120)	7.3 ± 1.4 (10)	2.4 ± 0.83 (-)	4.8 ± 1.0 (-)
SVT-40776	0.4 ± 0.09	38.5 ± 6.4	0.19 ± 0.09	0.37 ± 0.01	0.4 ± 0.17

Footnote: Membranes were obtained from *Receptor Biology Inc*. Values are expressed as the mean of, at least, two independent experiments, eight points per curve, each point performed in duplicate. Hill slopes of competition curves were not significantly different from unity. Where indicated, numbers in parentheses represent the values described in the literature.

Table 9. Selectivity shown by antagonists in radioligand binding assays at M_1-M_5 recombinant human muscarinic receptor subtypes.

COMPOUND	AFFINITY (Ki, nM)	RATIO			
	hM_3	**M_1/M_3**	**M_2/M_3**	**M_4/M_3**	**M_5/M_3**
Oxybutynin	1.6 ± 0.3 (0.7-2.0)	2.4	5.7	1.2	3.3
Tolterodine	4.13 ± 1.7 (3.4)	0.6	0.5	0.4	0.6
Darifenacin	3.05 ± 0.2 (1.2-4)	16.6	29.1	6.9	2.4
Solifenacin	7.3 ± 1.4 (10)	0.3	5.9	0.3	0.7
SVT-40776	0.19 ± 0.09	2.0	203.7	1.5	2.1

Footnote: Membranes were obtained from *Receptor Biology Inc*. Values are expressed as the mean of, at least, two independent experiments, eight points per curve, each point performed in duplicate. Where indicated, numbers in parentheses represent the values described in the literature. Ratios were obtained from the data of the previous table.

SVT-40776 exhibited the highest affinity, in the sub-nanomolar range (K_i= 0.19 nM), for the human M_3 muscarinic receptor, thus being the most potent ligand among all the reference compounds assayed. SVT-40776 exhibited higher affinity for the human M_3 receptor than for the rest of muscarinic receptor subtypes. Particularly, SVT-40776 displayed the highest selectivity (203-fold) for the M_3 *vs.*

the M_2 receptor subtype, in comparison with the other antagonists tested. Darifenacin also showed marked M_3 *vs.* M_2 selectivity (29.4-fold), while oxybutynin (5.7-fold) and solifenacin (5.9-fold) showed less selectivity for the M_3 receptor, and tolterodine completely lacked of selectivity.

1.2. Competition studies with [^{3}H]NMS

1.2.1. Characterization of SVT-40776 binding behaviour

To investigate the reversible nature of SVT-40776 binding compared with other antagonists, CHO-M_2 and CHO-M_3 cell membranes were preincubated with the compounds and further diluted in the presence of 0.5 nM [^{3}H]NMS. An antagonist concentration achieving more than 60% inhibition in the competition curves was chosen. As shown in Figure 16, [^{3}H]NMS binding was recovered after dilution in samples preincubated with SVT-40776, darifenacin or tolterodine, suggesting that these antagonists behaved as reversible ligands of both M_2 (Figure 15A) and M_3 (Figure 15B) receptors. As expected, the binding was not recovered after 4-DAMP mustard preincubation and dilution, clearly indicating an irreversible interaction with both receptors. Besides, the recovery of the [^{3}H]NMS binding after a 50-fold dilution was in good agreement with that found in the competition curves in all cases.

To elucidate the type of interaction of SVT-40776 with the human M_3 and M_2 receptors, [^{3}H]NMS saturation curves were performed in the presence of different antagonist concentrations. The presence of SVT-40776, darifenacin, solifenacin and tolterodine produced concentration-dependent rightward shifts of [^{3}H]NMS saturation curves in M_3 receptor when compared to that obtained in the absence of antagonists. These displacements were accompanied by a concomitant increase in the K_d value without significantly changing the B_{max} value in the case of SVT-40776 (Table 10), darifenacin, solifenacin and tolterodine for the M_3 receptor (Table 11), which was compatible with a competitive binding. As expected, 4-DAMP mustard induced a concentration-dependent reduction in the B_{max} value without modifying the K_d parameter, thus confirming its irreversible behaviour. Additionally, [^{3}H]NMS saturation studies were carried out with CHO-M_2 cell membranes preparations to further examine

SVT-40776 binding features. As shown in Table 10, SVT-40776 displaced [^{3}H]NMS from the binding site, increasing its K_d without any change in B_{max} value. This indicated that the compound behaved as a competitive antagonist of the M_2 ACh receptor too. However, this effect was only observed from 10 nM concentration up, since at lower concentrations no changes in K_d and B_{max} parameters were detected.

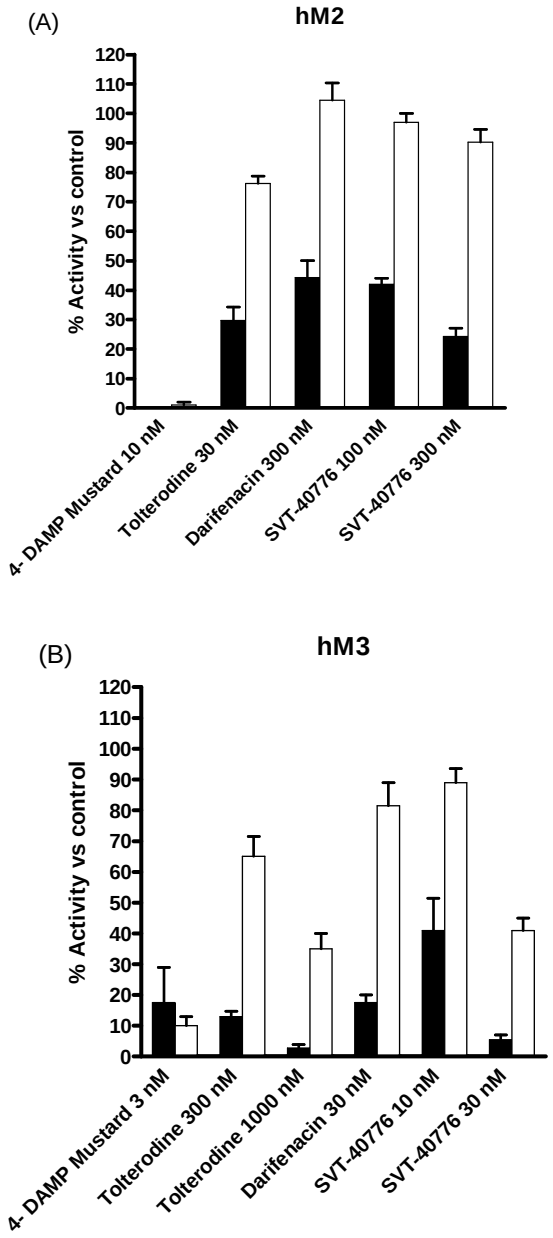


Figure 15. Reversibility of the interaction of mACh antagonists with hM_2 and hM_3 receptors. Human M_2 **(A)** and M_3 **(B)** receptors were preincubated for 3 h with the antagonists at the concentrations indicated. Then, ligand displacement was achieved after a 50-fold dilution (open bars). The free binding sites were determined by [^{3}H]NMS (0.5 nM) binding and compared with those without dilution (closed bars). Results are expressed as the mean ± S.E.M. of 2-3 independent experiments.

Table 10. Effect of SVT-40776 on [^{3}H]NMS binding parameters to hM_2 and hM_3 receptors

SVT-40776 (nM)	hM_3		hM_2	
	K_D	B_{max}	K_D	B_{max}
0	0.69±0.05	1364±32	1.03±0.1	4257±837
0.1	0.78±0.02	1291±103		
0.3	0.91±0.07	1251±118		
1	1.7±0.05**	1447±159		
3	3.9±0.5*	1458±43		
10	9.5±0.2**	1420±202	1.2±0.06	3930±83
30	27.5±14**	1493±720	1.5±0.3	4230±363
100			3.6±0.2**	4393±480
300			7.9±0.3*	4446±1629
1000			24±0.8**	4369±827
3000			63±0.7***	5208±607

Footnote: Membranes obtained from CHO-K1 cells stably expressing human muscarinic receptors were used. K_d is expressed in nM and B_{max} in fmols/mg. Data are expressed as the mean ± S.E.M. of 2 independent experiments. Student's t test: *p<0.05 and **p<0.01 *vs.* control curves in the absence of antagonist.

Table 11. Effect of mACh receptor antagonists on [^{3}H]NMS binding parameters to the hM_3 receptor

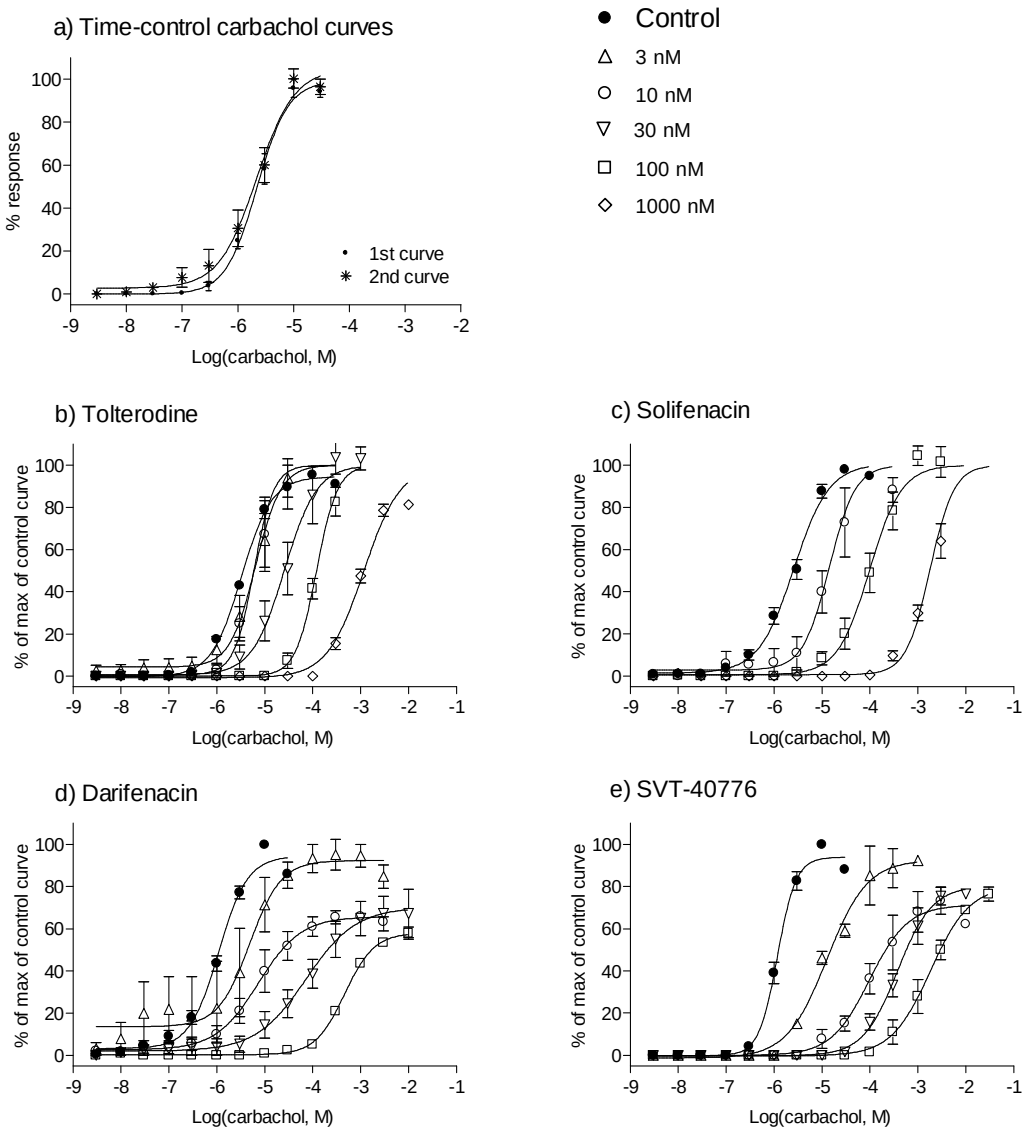
Concentration (NM)	DARIFENACIN		SOLIFENACIN		TOLTERODINE		4-DAMP MUSTARD	
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}
0	0.71±0.1	1463±111	1.1±0.7	1571±239	1.99±1.4	1639±253	1.4±0.5	1527±293
0.1	-	-	0.6±0.02	1321±93	-	-	1.5±0.4	1365±129
0.3	-	-	0.5±0.02	1293±95	-	-	1.3±0.1	1212±17
1	2.1±1.4	1526±24.4	0.7±0.09	1328±88	0.6±0.08	1247±302	1.3±0.4	773±203
3	1.2±0.5	1411±123	0.8±0.1	1278±110	-	-	1.0 ± 0.5	330±141*
10	1.5±0.3	1263±280	1.1±0.3	1240±101	0.9±0.15	1491±302	-	-
30	2.9±0.05**	1128±328	-	-	3.3±1.7	1525±408	-	-
60	7.7±1.1*	1171±396	-	-	-	-	-	-
100	11.9±4.0*	1488±615	8.4±3.7**	1499±358	8.4±3.2	1358±309	-	-
200	-	-	-	-	12±4.2*	1328±147	-	-
300	-	-	-	-	27±3.0**	1530±414	-	-
600	-	-	-	-	52±1.4**	1278±351	-	-

Footnote: Membranes obtained from CHO-K1 cells stably expressing human muscarinic receptors were used. K_d is expressed in nM and B_{max} in fmols/mg. Values are expressed as the mean ± S.E.M. of 2-4 independent experiments. Student's t test: *p<0.05 and **p<0.01 *vs.* control curves in the absence of antagonist.

2. ISOLATED TISSUE FUNCTIONAL STUDIES

2.1. Effect of SVT-40776 on mice isolated bladder *in vitro*

CCh induced concentration-dependent contractions of mice urinary bladder smooth muscle. The first curve yielded a mean pEC50 = 5.66 (5.58-5.74) with maximum responses of 1.19 ± 0.22 g. A consecutive additional CCh concentration-response curve within this preparation in the absence of antagonist yielded a mean pEC50 = 5.66 (5.50-5.82) with an Emax of 1.22 (1.03-1.41) g (n = 4). Thus, two consecutive concentration-effect curves to CCh were constructed in the same tissue with no significant change in the agonist potency and maximum response (Figure 16a). Antagonists concentration-dependently shifted the CCh concentration-response curve to the right. Methoctramine and pirenzepine did show a pA_2 compatible with its affinity on the M_3 receptor. Both 4-DAMP and 4-DAMP mustard presented a pA_2 also in agreement with its affinity for the M_3 receptor, also alowing to visualize clearly the irreversibility of the mustard on the profile of the pA_2. While tolterodine and solifenacin did not significantly alter maximum CCh response (Figure 16b,c), darifenacin exposure clearly reduce Emax (from 66% at 3 nM to 58% at 100 nM) (Figure 16d). SVT-40776 also reduced slightly the Emax to 71% at 10 nM and 80% at 100 nM (Figure 16e). 60 min incubation time was used for all compounds before carring out the secong CCh curve. However, an experiment evaluating the effect of different incubation times for SVT-40776 at one concentration was performed (Figure 17). The results showed no differences from 15 min to 60 min incubation times. Analysis of the shifts of the CCh concentration-response curves by all antagonists yielded Schild correlation coefficients (r^2 values) near to unit. Most slopes were also close to unit, except for SVT-40776, darifenacin and 4-DAMP mustard. pA2 values and slopes are compared in Table 12.


a) Time-control carbachol curves
% response
Log(carbachol, M)
1st curve
2nd curve
Control
3 nM
10 nM
30 nM
100 nM
1000 nM
b) Tolterodine
c) Solifenacin
d) Darifenacin
e) SVT-40776
% of max of control curve
% of max control curve

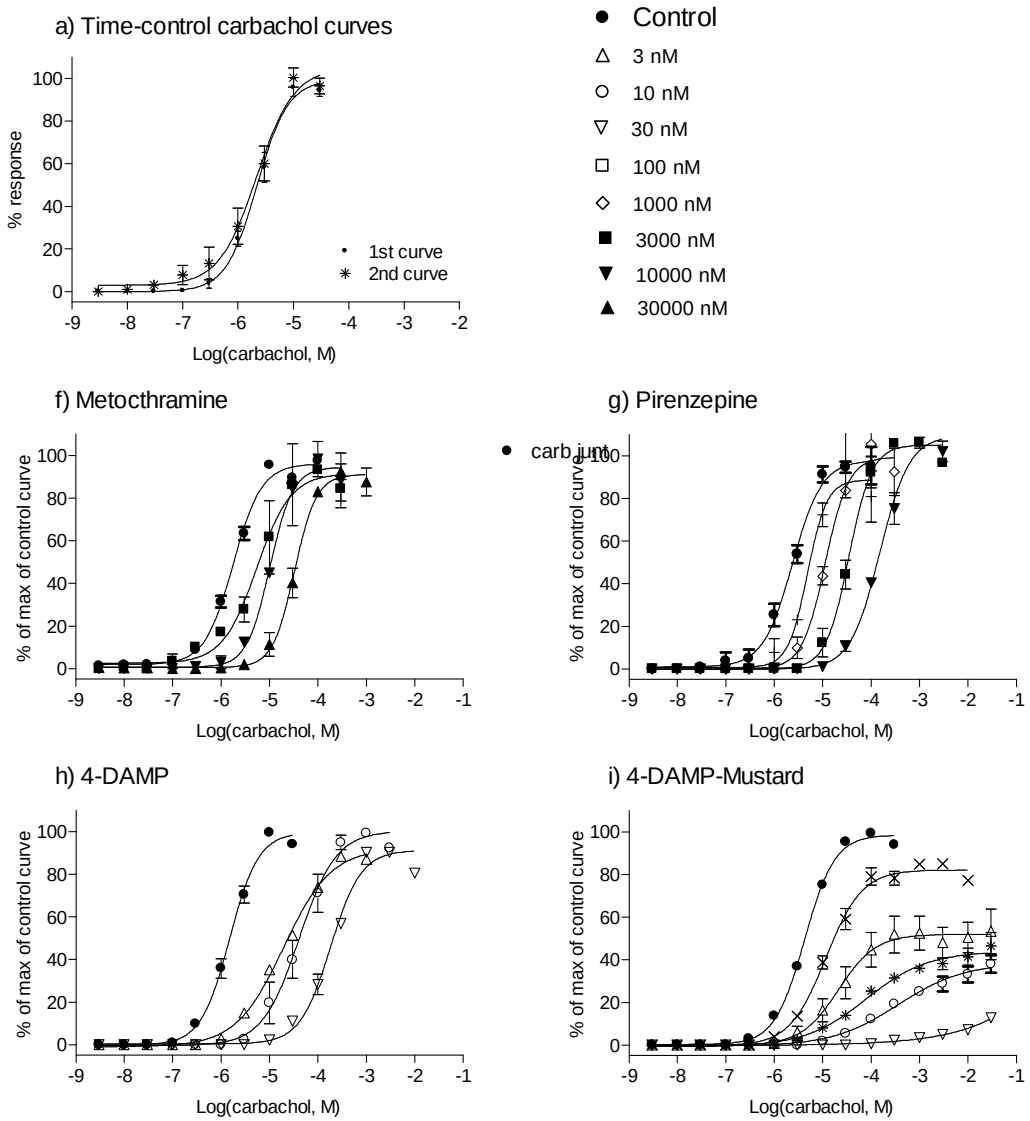


Figure 16. Effects of tolterodine (b), solifenacin (c), darifenacin (d) and SVT-40776 (e) , methoctramine (f), pirenzepine (g), 4-DAMP (h) and 4-DAMP-mustard (i) on the cumulative consecutive concentration-response curves to CCh (a) on mouse urinary bladder. Direct contractile effects were expressed as percentages of the maximum response of the control curve. Data are expressed as means ± SEM, n= 4-8 animals/concentration. A single concentration of antagonist was applied to each tissue.

Table 12. Effect of SVT-40776 and competitors on CCh-induced bladder contractions.

COMPOUND	BLADDER DETRUSOR pA_2 (CI)	SLOPE
Methoctramine	6.30 (6.15-6.49)	0.80±0.08
Pirenzepine	6.48 (6.42-6.54)	1.20±10.04
4-DAMP	9.58 (9.40-9.66)	0.99±0.21
4-DAMP Mustard	9.05 (8.78-9.32)	1.69±0.35
Tolterodine	8.44 (8.2-8.6)	1.06 ±0.10
Darifenacin	8.81 (8.2-9.4)	1.27±0.52
Solifenacin	8.57 (8.2-8.9)	1.11 ±0.06
SVT-40776	9.55 (9.2-9.8)	1.36±0.12

Footnote: n= 4-6 animals for each antagonist concentration

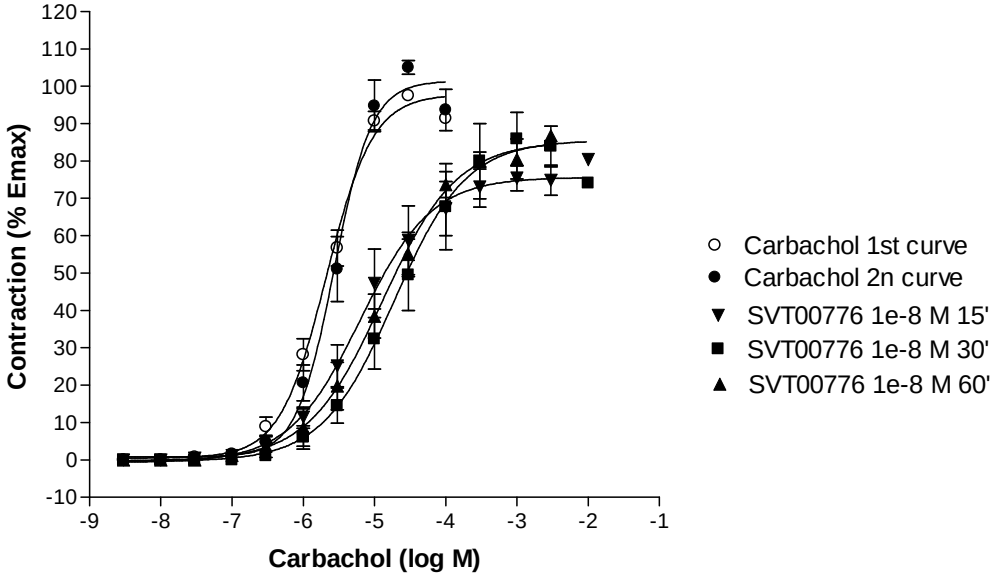


Figure 17. Effects of different times of incubation for SVT-4077 on the cumulative consecutive concentration-response curves to CCh on mouse urinary bladder. Direct contractile effects were expressed as percentages of the maximum response of the control curve. Data are expressed as means ± SEM, n= 8 animals/concentration. A single concentration of antagonist was applied to each tissue.

2.2. Effect of SVT-40776 on mice isolated bladder *ex vivo*

The pEC_{50} obtained for CCh controls in the *ex vivo* protocol at 1h, 3h, 5h and 24h was 4.74 (4.53-4.95), 5.19 (5.13-5.25), 4.93 (4.64-5.22) and 5.28 (5.09-5.47) respectively.

First step was to carry out a time course in the bladder *ex vivo* protocol to determine the time course of the activity. Thus, a clear time depending response was observed for all the compounds that most probably correlated with its pharmacokinetic and tissue distribution in mice. SVT-40776 showed efficacy from 1h post – dosing up to 24h. Comparing efficacy at different time points SVT-40776 was the most potent compound at 1h, 3h and 24h. Thus, SVT-40776 shows that it is rapidly distributed to the target organ and it remains in the bladder for a very long time. Comparatively darifenacin did not produce any inhibition till 3h post-dosing. At 1h there was a complete lack of activity. However darifenacin showed to be the most potent antagonist at the 5h post-dosing observation. Taking into account that tolterodine is highly metabolised in mice and its metabolites are active (see Chapter 1.2.), the activity seen at 3h are suspected to be due to the parental compound plus the metabolites, fact that do not occur with SVT-40776, which metabolise to one major metabolite that it is not active (data not shown).

Table 13. Time-course of orally administered SVT-40776 and several competitors on CCh-induced bladder contractions

Detrusor *ex vivo* pA_2-ED mg/kg p.o. (slope)	**Pre-treatment time**			
	1h	**3h**	**5h**	**24h**
Tolterodine	3.1 (0.7)	0.66 (0.68)	11.3 (1.0)	50-100
Darifenacin	>10	1.55 (1.11)	0.9 (0.83)	95 (1.5)
Solifenacin	6.8 (0.6)	1.39 (1.19)	7.6 (0.8)	127.1 (0.4)
SVT-40776	3.4 (2.1)	0.72 (1.42)	1.8 (1.4)	72.8 (4.2)

Footnote: Mice were sacrificed at 1h, 3h, 5h or 24h after receiving a single oral dose of antagonists or vehicle. n= 4-6 for each antagonist dose, 3-5 doses per compound.

As 3h showed to be the best time for most compounds, an extensive work was carried out specifically at this time point. Thus, detrusor smooth muscle contractility from control animals sacrificed 3 h after receiving an oral dose of vehicle did not show any significant differences compared with non-treated animals. CCh induced concentration-response curves yielding pEC50 = 5.19 (5.13-5.25) (n = 22) (

Figure **18**a). This value was assigned as control pEC50, in order to compare it with pEC50 values from antagonist-treated animals. KCl produced a maximum effect similar to CCh of 1.16 (0.98-1.34) g. Right shifting of the CCh response curves by tolterodine, solifenacin, darifenacin and SVT-40776 were obtained (

Figure **18**b-e). Darifenacin at 50 mg/kg reduced the Emax. All antagonists yielded Schild correlation coefficients (r^2 values) near to unity. However, steep slopes were seen for darifenacin and SVT-40776 (superior to 1.2) compared with the other two antagonists. pA2-ED values and slopes are compared in Table 14.

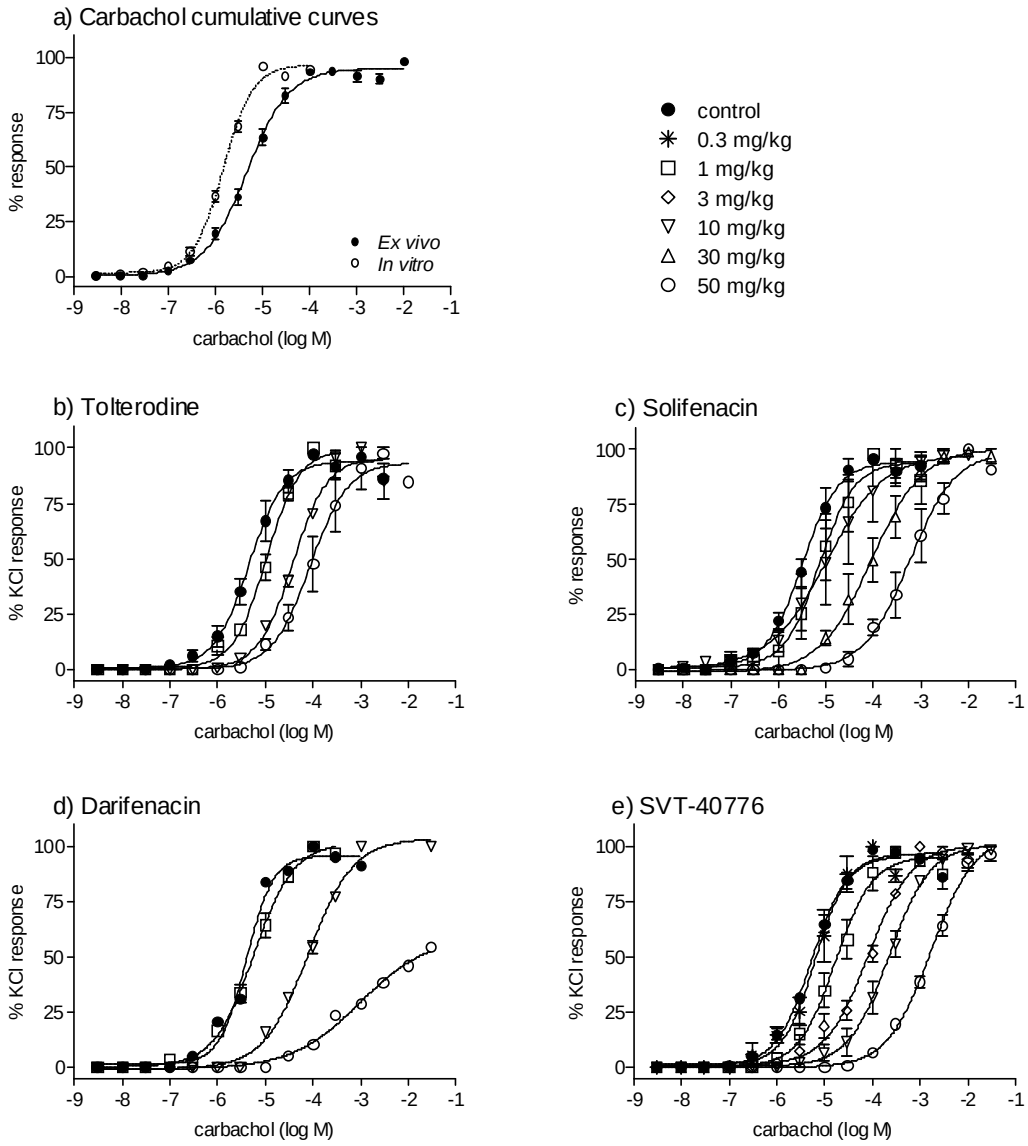


Figure 18. Effects of oral administration of muscarinic receptor antagonists on the concentration-response curves of CCh-induced contractions in mouse urinary bladder. Data are expressed as means ± SEM, n= 4-6 animals/dose.

Table 14. Effect of oral SVT-40776 on CCh-induced isolated bladder contractions

COMPOUND	BLADDER DETRUSOR $pA\text{-}ED_{50}$ (IC) (mg/kg po)	SLOPE
Tolterodine	0.66 (0.40-1.0)	0.68±0.05
Darifenacin	1.55 (0.35-6.71)	1.11±0.06
Solifenacin	1.39 (0.46-4.20)	1.19±0.7
SVT-40776	0.72 (0.41-1.10)	1.42±0.1

Footnote: Mice were sacrificed at 3h after receiving a single oral dose of antagonists or vehicle.n= 4-6 for each antagonist dose, 3-5 doses per compound.

2.3. Effect of SVT-40776 on dog isolated bladder *ex vivo*

Detrusor smooth muscle from control animals 1h and 3 h after receiving an oral dose of vehicle did not show any significant differences compared with non-treated animals. CCh induced concentration-response curves yielding pEC50 = 5.82 (5.89-5.74) (

Figure 19a). This data was assigned as control pEC50 in order to compare it with pEC50 from antagonist-treated animals. Also in this Figure a comparation of the CCh-induced concentration-response curve in mice is shown in order to compare the response in both species. No significant differences wre observed between species. Right shifting of the CCh response curves for SVT-40776 were obtained (

Figure 19b). SVT-40776 yielded a Schild regression line close to unit. pA_2-ED values are compared in Table 15.

A single administration of SVT-40776 was able to inhibit dog detrusor CCh-induced contractions after an oral administration, being more potent after 3h post-dosing than after 1h. These results showed that SVT-40776 was absorbed and clearly distributed on the bladder 1h after oral dosing producing a clean blockade of carcachol–induced contractions on the muscle. This effect increased 3h post-dosing suggesting a prolonged duration of action of the compound on urinary bladder.

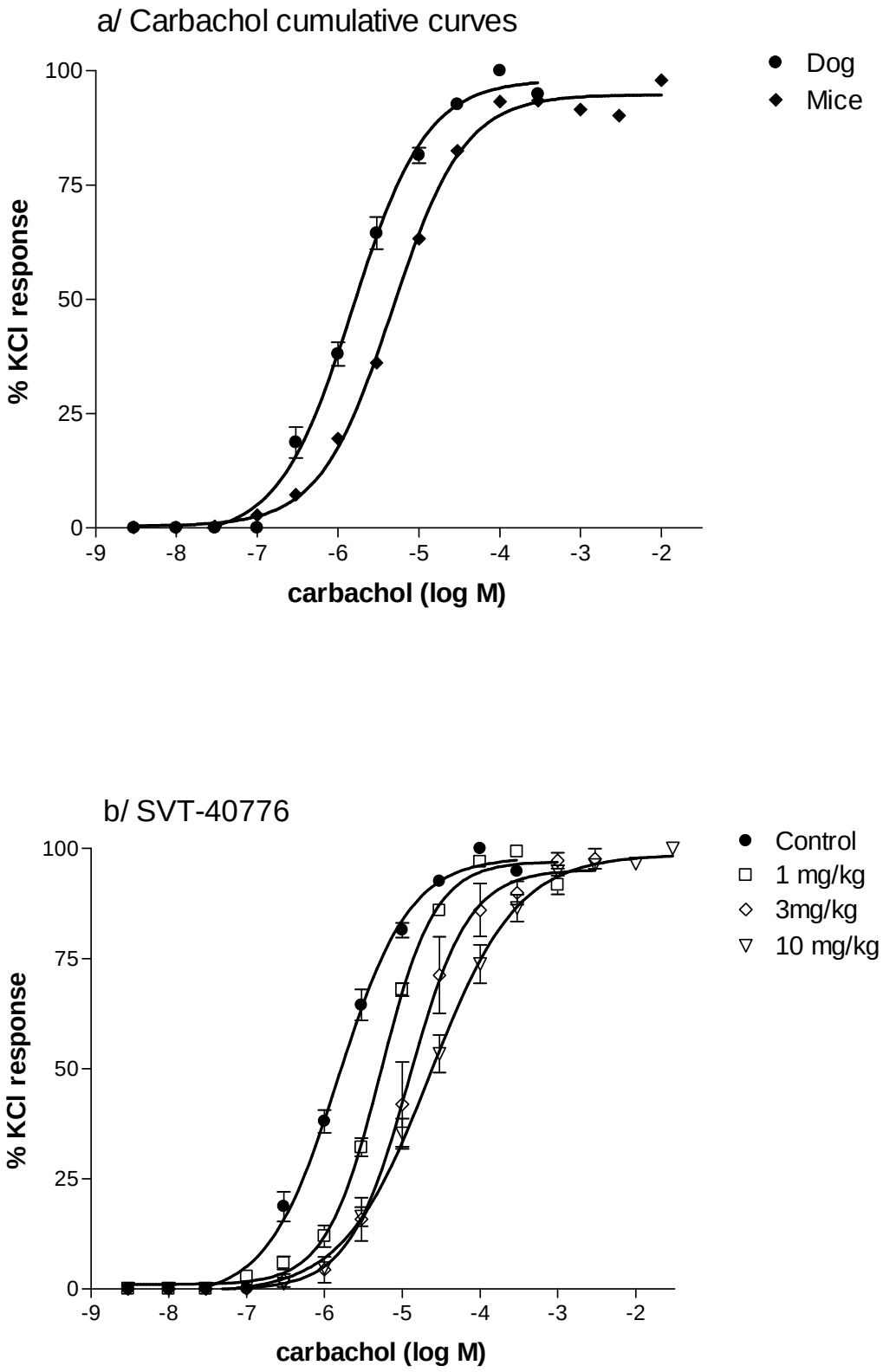


Figure 19. Effects of oral administration of SVT-40776 in dog urinary bladder (a) Comparative CCh response in mice and dog ex vivo bladder (b) SVT-40776 concentration-response curves of CCh-induced contractions. Data are expressed as means ± SEM, n= 4 animals/dose.

Table 15. pA_2-ED of SVT-40776 in dogs.

POST-ADMINISTRATION TIME	
1h	**3h**
pA_2-ED (mg/kg po) (slope)	**pA_2-ED (mg/kg po) (slope)**
1.09 (1.23±0.44)	0.31 (0.82±0.34)

Footnote: Dog samples were obtained at 1h or 3h after receiving a single oral dose of antagonists or vehicle. n= 4 for each antagonist dose.

3. WHOLE ANIMAL FUNCTIONAL STUDIES

3.1. Effect of SVT-40776 on anaestethised guinea pig urodynamia

Isovolumetric model

Figure 20 shows a representative trace of spontaneous contractions before and after consecutive saline or antagonist bolus. Bladder contraction amplitude was measured during the 15-min period between doses and the percentage of variation of bladder contraction amplitude was calculated *vs.* baseline. Intravenous administration of SVT-40776, oxybutynin, tolterodine, darifenacin and solifenacin changed bladder contraction amplitude in a dose-dependent manner (

Figure 21). SVT-40776 revealed itself as the most potent muscarinic antagonist evaluated in the guinea pig *in vivo* model, inhibiting 25% of spontaneous bladder contractions at very low doses, such as 6.9 µg/kg. Calculated effective doses of darifenacin, oxybutynin, solifenacin and tolterodine were 3, 11, 12 and 17-fold higher than that of SVT-40776, respectively (Table 16). Tolterodine exhibited an extremely low urinary selectivity in this assay (2.4-fold).

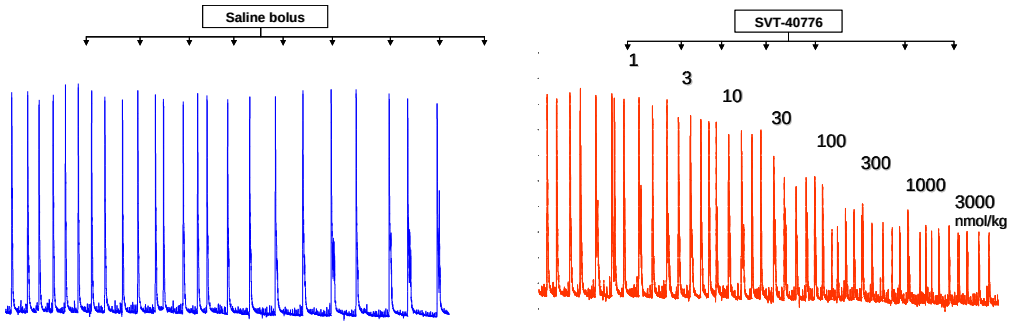


Figure 20. Left/ *In vivo* cystograph showing guinea pig bladder spontaneous contractions. Right/ Inhibitory effect of SVT-40776 (cumulative concentration curve) on those spontaneous contractions.

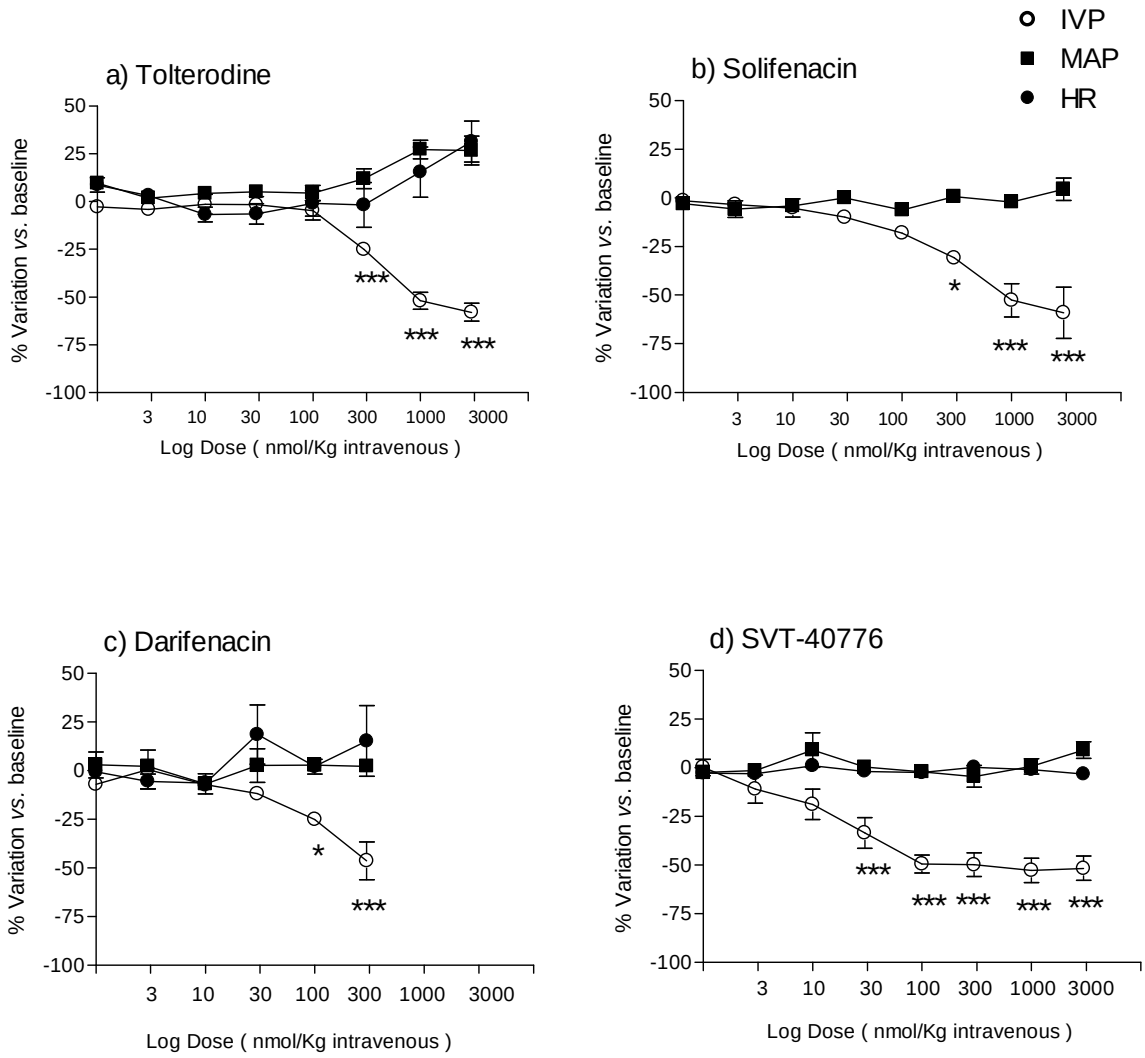


Figure 21. Effect of intravenous administration of muscarinic receptor antagonists on bladder contraction amplitude in guinea pig. Bladder contraction amplitude was measured during the 15-min period between doses and the percentage of variation of bladder contraction amplitude was calculated versus baseline. IVP =intravesical pressure; MAP: mean arterial pressure; HR: heart rate. Student's t test: *p<0.05, **p<0.01 and ***p<0.001 vs. control

Table 16. (a) Effect of SVT-40776 on bladder and arterial pressure *in vivo.*

Compounds	BLADDER PRESSURE (BP) INHIBITION		ARTERIAL PRESSURE (AP) INCREASE	
	ED_{25}		ED_{25}	
	ng/kg	nmols/kg	ng/kg	nmols/kg
Tolterodine	97.55	299.2	267.3	820
Darifenacin	22.69	53.2	>500	>1000
Solifenacin	72.82	200.9	>1000	>3000
SVT-40776	6.97	17.1	>1000	>3000

(b) Selectivity ratios comparing arterial vs. bladder pressure

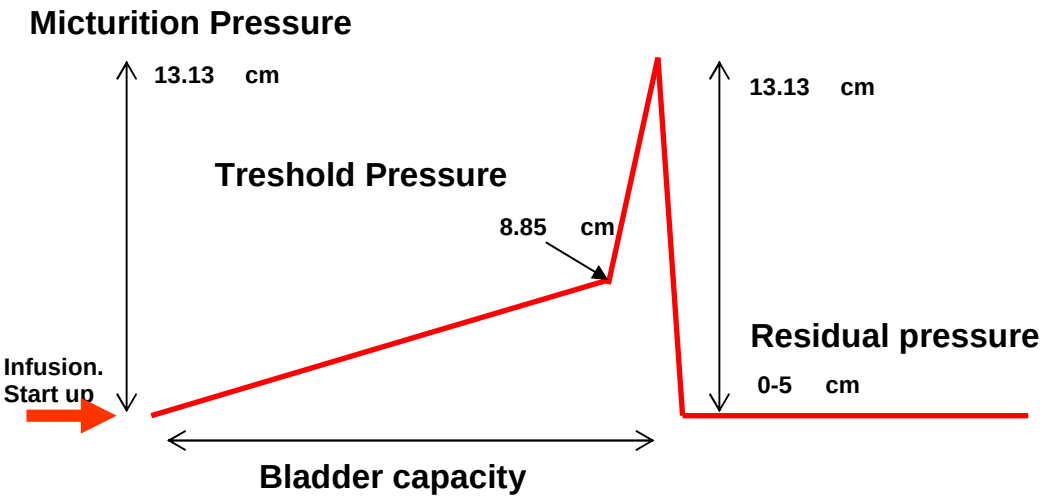
Compounds	SELECTIVITY RATIO AP/BP
Tolterodine	2.7
Darifenacin	22.0
Solifenacin	13.7
SVT-40776	143.5

Footnote: Where there was not AP calculated values, the maximum dose tested without effect was choosed to estimate the ratio.

Cystometry

The first step was to characterize the response to bladder filling in non-treated animals (n=14). Once the saline response was well established, the compounds were tested. We used 6 animals per compound and dose and matching controls were also running in all the experiments so 85 animals were accumulated as controls (saline micturition cycles). A scheme (a) and a real control (b) cystograph is shown (Figure 22).

a/



b/

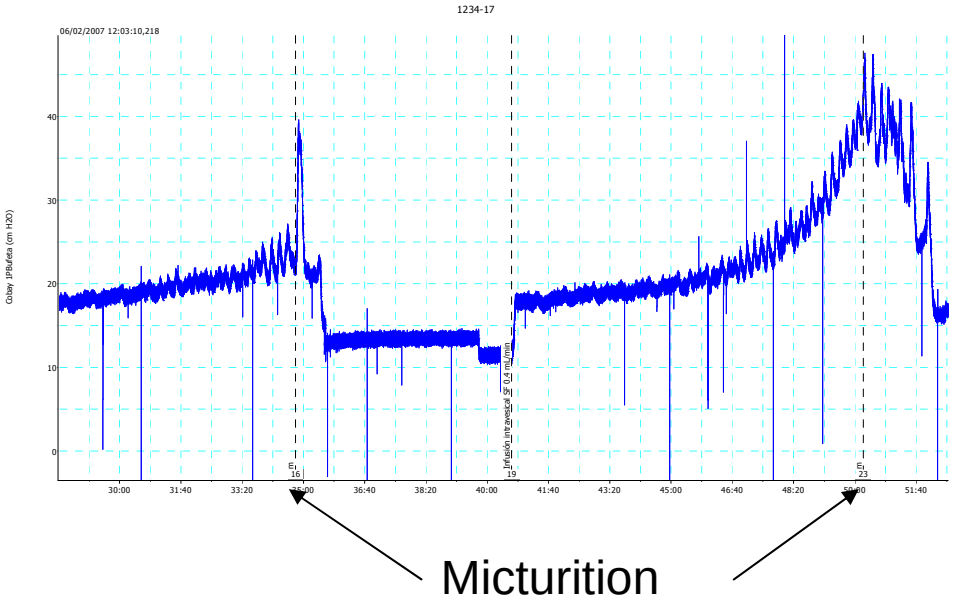


Figure 22. A scheme (a) and an *in vivo* (b) cystograph of a guinea pig bladder micturiton induced by continuous saline infusion. (b) Effect on the higher dose of SVT-40776 can be appreciated in the last micturition cycle.

Animals produced very repetitive micturition cycles, obtaining no statistically significant differences between the parameters evaluated (Micturition Pressure (MP), Bladder Capacity (BC), Treshold Pressure (TP), Resting Pressure (RP)) between cycles (Table 17). A fifth cycle was studied in 8 animals to evaluate the resistance of the bladder. The results can be seen in Table 18. Interestingly, there were also no significant changes between the fifth micturition cycle and the previous ones. Arterial pressure was maintained inalteraded during the whole procedure (see Table 17 and Table 18).

Table 17. Treshold pressure, micturition pressure and bladder capacity of non-treated animals in the four consecutive micturition cycles.

	TP cm H_2O	MP cm H_2O	BC mL	RP cm H_2O	MAP cm H_2O
First micturition					
	8.52 ±1.27	17.41±1.14	2.93±0.22	5.69±0.8	7.71±1.22
Second micturition					
	7.98±1.01	16.09±0.93	2.85±0.22	7.99±2.23	8.28±1.31
Third micturition					
	6.58±0.56	14.76±0.75	2.92±0.25	5.35±0.88	5.89±1.0
Fourth micturition					
	6.82±0.6	14.08±0.92	2.75±0.27	5.79±0.78	5.80±1.1

Footnote: mean ± SEM (n= 14) ; Micturition Pressure (MP), Bladder Capacity (BC), Treshold Pressure (TP), Resting Pressure (RP)

Table 18. Data obtained in the fifth consecutive micturition cycle.

	TP cm H_2O	MP cm H_2O	BC mL	RP cm H_2O
First micturition				
	7.92±1.8	15.28±1.32	2.47±0.21	6.12±2.18

Footnote: mean ± SEM (n= 8); Micturition Pressure (MP), Bladder Capacity (BC), Treshold Pressure (TP), Resting Pressure (RP)

As the effect of the compound is calculated measuring the delta between the third and the fourth micturition cycle, the delta for the non-treated animals should be near to 0 as it would indicate a similar response between the third and the four

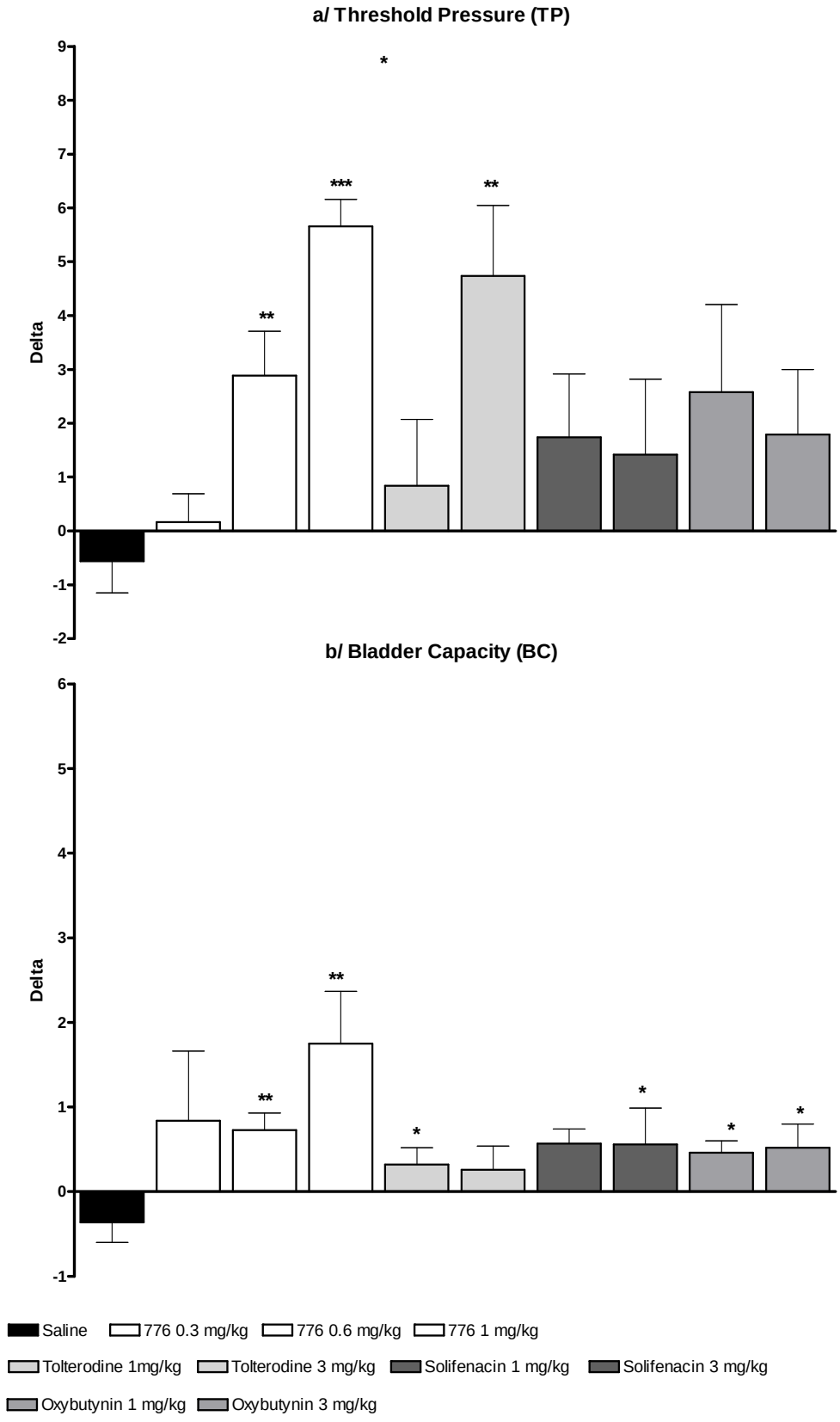
cycle. Deltas significantly different from 0 would indicate a difference in the fourth cycle. The non- treated animal deltas are shown in Table 19.

Table 19. Delta between parameters when comparing the fourth *vs.* the third micturition cycle

Fourth vs. third micturition cycle	TP cm H_2O	MP cm H_2O	BC mL	RP cm H_2O
Delta	0.24	-0.68	-0.17	0.44

Footnote: n =8; no significant differences at any parameter¸ Micturition Pressure (MP), Bladder Capacity (BC), Treshold Pressure (TP), Resting Pressure (RP)

SVT-40776, tolterodine, oxybutynin and solifenacin have been tested in this model in a dose range between 0.3-3 mg/kg i.v. The results are shown in the following graphs (Figure 23).


a/ Threshold Pressure (TP)
Delta
9
8
7
6
5
4
3
2
1
0
-1
-2
*
***
**
**
b/ Bladder Capacity (BC)
Delta
6
5
4
3
2
1
0
-1
**
**
*
*
*
*
Saline
776 0.3 mg/kg
776 0.6 mg/kg
776 1 mg/kg
Tolterodine 1mg/kg
Tolterodine 3 mg/kg
Solifenacin 1 mg/kg
Solifenacin 3 mg/kg
Oxybutynin 1 mg/kg
Oxybutynin 3 mg/kg

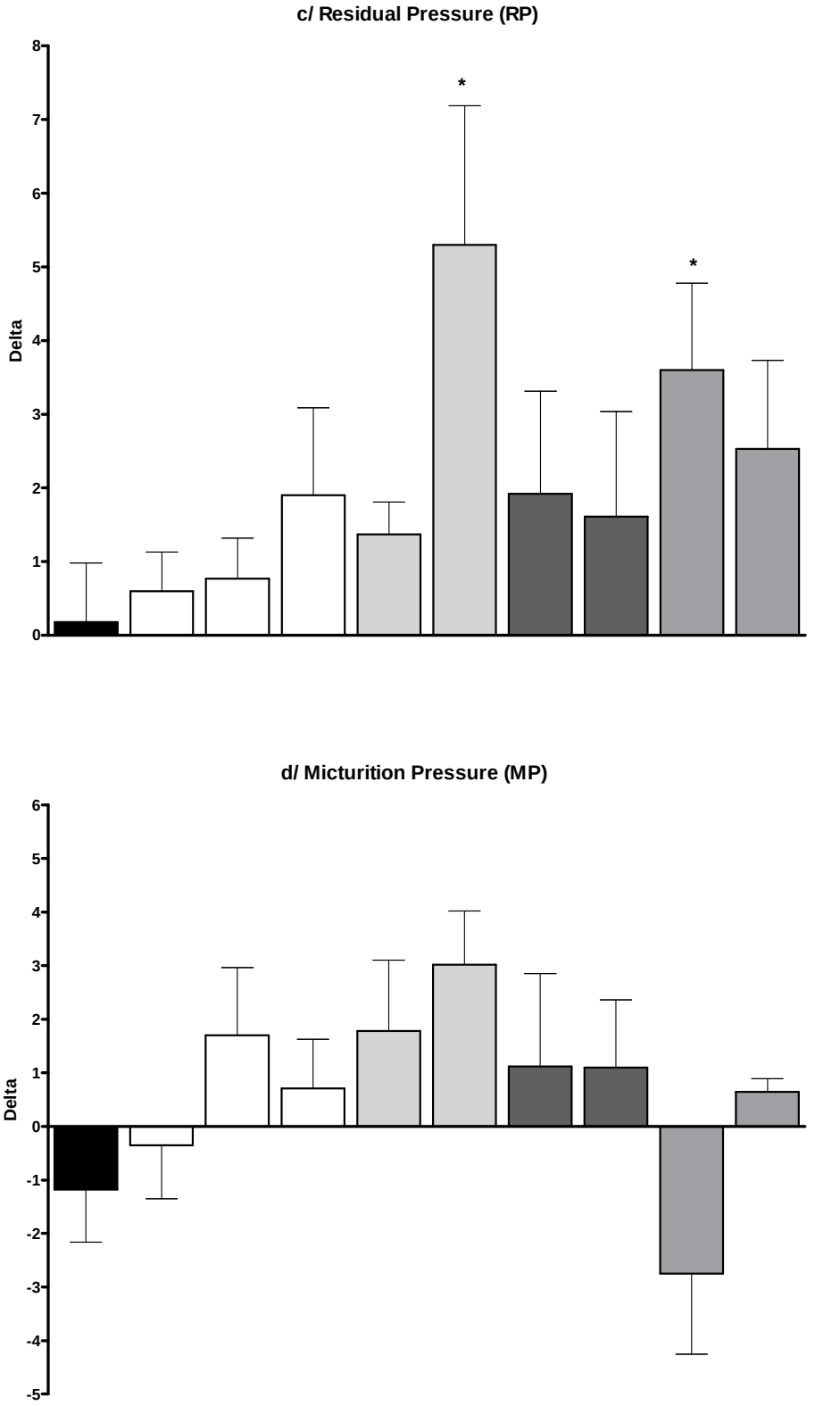

c/ Residual Pressure (RP)
Delta
8
7
6
5
4
3
2
1
0
*
*
d/ Micturition Pressure (MP)
Delta
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
Saline
776 0.3 mg/kg
776 0.6 mg/kg
776 1 mg/kg
Tolterodine 1mg/kg
Tolterodine 3 mg/kg
Solifenacin 1 mg/kg
Solifenacin 3 mg/kg
Oxybutynin 1 mg/kg
Oxybutynin 3 mg/kg

Figure 23. Effects on a/TP, b/BC c/RP and d/MP for all the compounds evaluated in the guinea pig cystometry. Student's t test: *p<0.05, **p<0.01 and ***p<0.001 *vs.* control

SVT-40776 increased dose-dependently the TP and the BC, being significant from 0.6 mg/kg for both parameters. MP was not affected.

This increase on TP and BC was observed in all the antimuscarinics tested, although not significant for TP but for BC for solifenacin and oxybutynin at all doses tested. SVT-40776 was the most potent compound compared with the competitors. Solifenacin and oxybutynin were not as effective as SVT-40776 up to 3 mg/kg.

RP is an indirect measurement of residual volume. SVT-40776 was the only compound that showed a window between the effect on TP and the effect on RP. At 1 mg/kg the effect on TP was very significant but no effect was seen in the RP. However, tolterodine did not show window. It did not have significant effects on TP when was affecting the RP. Oxybutynin did have even a more essential effect. At doses of 1 mg/kg, with a tendency to increase TP did increase RP very significantly. Thus, in Table 20 the different profile at the highest dose tested for each compound is shown.

Table 20. Delta values obtained at the highest dose tested for each antagonist

Fourth vs. third micturition cycle	Dose (mg/kg i.v.)	TP cm H_2O	MP cm H_2O	BC mL	RP cm H_2O	MAP cm H_2O
Tolterodine	3	4.7±1.3**	3.02±1.0	0.26±0.28	5.3±1.9*	33.3±4.8***
Oxybutynin	3	2.6±1.6	0.64±0.23	0.52±0.28*	2.53±1.2	10.1±3.2
Solifenacin	3	1.4±1.4	1.1±1.3*	0.56±0.43*	1.6±1.4	28.8±3.1***
SVT-40776	1	5.7±0.5***	0.71±0.92	1.8±0.6**	1.9±1.2	18.4±4.3*

Footnote: n = 6-8 animals/dose. Micturition Pressure (MP), Bladder Capacity (BC), Treshold Pressure (TP), Resting Pressure (RP). Student's t test: *p<0.05, **p<0.01 and ***p<0.001 *vs.* control.

4. FUNCTIONAL SELECTIVITY STUDIES

4.1. Effect of SVT-40776 on mice isolated atria *in vitro*

In atria preparations, CCh induced concentration-dependent negative inotropism and cronotropism of spontaneous beating right atria. Only cronotropism was measured. Two consecutive concentration–effect curves to CCh could be constructed in the same tissue with no significant change in the agonist potency and maximum response. First and second curves yielded pEC50 of 6.55 (6.39-6.71) and 6.29 (6.03-6.55) respectively (n = 4). Maximum effect was also maintained in the second curve, which was the complete beating inhibition (Figure 24a). The four antagonists tested shifted the CCh curve dose-dependently to the right (Figure 24b). All antagonists yielded Schild correlation coefficients (r^2 values) near to unity. pA2 values and slopes are compared in Table 21a. A comparative of atria vs. detrsusor pA2 can be seen in Table 21b.

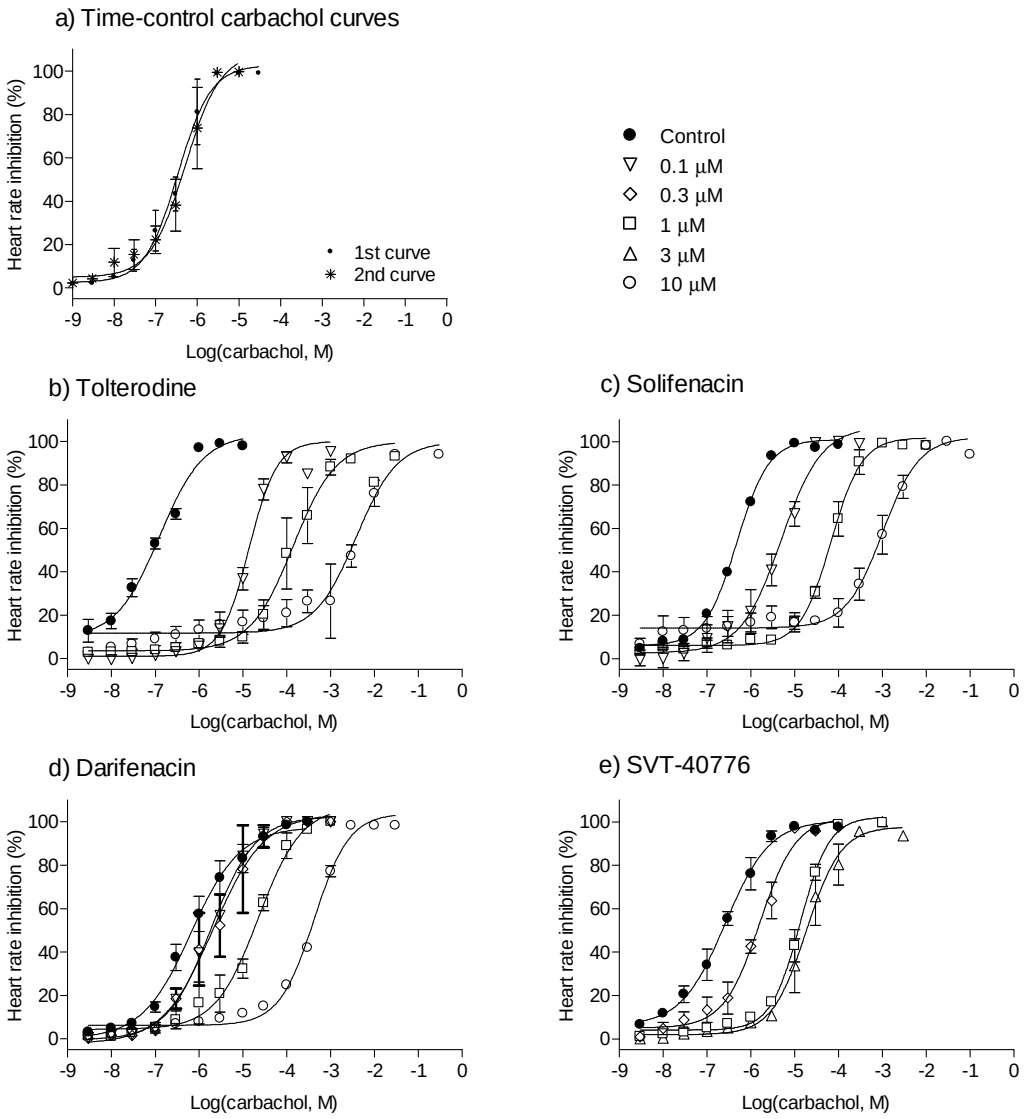


Figure 24. Effects of tolterodine (b), solifenacin (c), darifenacin (d) and SVT-40776 (e) on the cumulative consecutive concentration-response curves to CCh (a) on mouse atria. Direct contractile effects were expressed as percentages of the maximum response of the control curve. Data are expressed as means ± SEM, n= 4-6 animals/concentration. A single concentration of antagonist was applied to each tissue.

Table 21a Effect of SVT-40776 on CCh-induced heart contractions.

COMPOUND	ATRIA pA_2 (CI)	SLOPE
Methoctramine	8.36 (7.29-9.42)	0.95±0.11
Tolterodine	8.53 (7.8-9.2)	1.24±0.17
Darifenacin	7.27 (7.2-7.4)	1.26±0.02
Solifenacin	7.81 (7.7-7.8)	1.20±0.02
SVT-40776	7.25 (7.0-8.1)	1.15±0.45

Table 22b Bladder vs. Atria ratios

COMPOUND	pA_2 (CI)		SELECTIVITY RATIO HA/BD
	Bladder Detrusor (BD	Heart Atria (HA)	
Tolterodine	8.44 (8.2-8.6)	8.36 (7.29-9.42)	0.79
Darifenacin	8.81 (8.2-9.4)	8.53 (7.8-9.2)	31.6
Solifenacin	8.57 (8.2-8.9)	7.27 (7.2-7.4)	6.3
SVT-40776	9.55 (9.2-9.8)	7.81 (7.7-7.8)	199

Footnote: Values are expressed as mean ± S.E.M.., n= 4-8 animals per each antagonist concentration.

4.2. Effect of SVT-40776 on mice isolated atria *ex vivo*

Atria from control animals sacrificed 3 h after receiving an oral dose of vehicle did not exhibit any different behaviour compared with non treated animals. CCh induced concentration-response curves yielding pEC50 = 6.55 (6.37-6.73) (n = 28) (Figure 25a). This data was assigned as control pEC50, in order to compare it with antagonist-treated animal pEC50. In this protocol, as in the *in vitro* atria,

maximum effect was observed when complete inhibition of beating was obtained. Tolterodine and solifenacin dose-dependently shifted CCh curves to the right. Darifenacin exhibit less potency than tolterodine and solifenacin. SVT-40776 did not induce any relevant displacement of CCh curves to the right up to 30 mg/kg (Figure 25b). At 30 mg/kg its shift did not overcome two folds pED50 of control. Thus, the pA2-ED was calculated by extrapolation in order to calculate a ratio. All antagonists yielded Schild correlation coefficients (r^2 values) near to unity. pA2-ED values and slopes are compared in Table 23a. A comparative atria vs. detrusor pA2-ED can be seen in Table 23b.

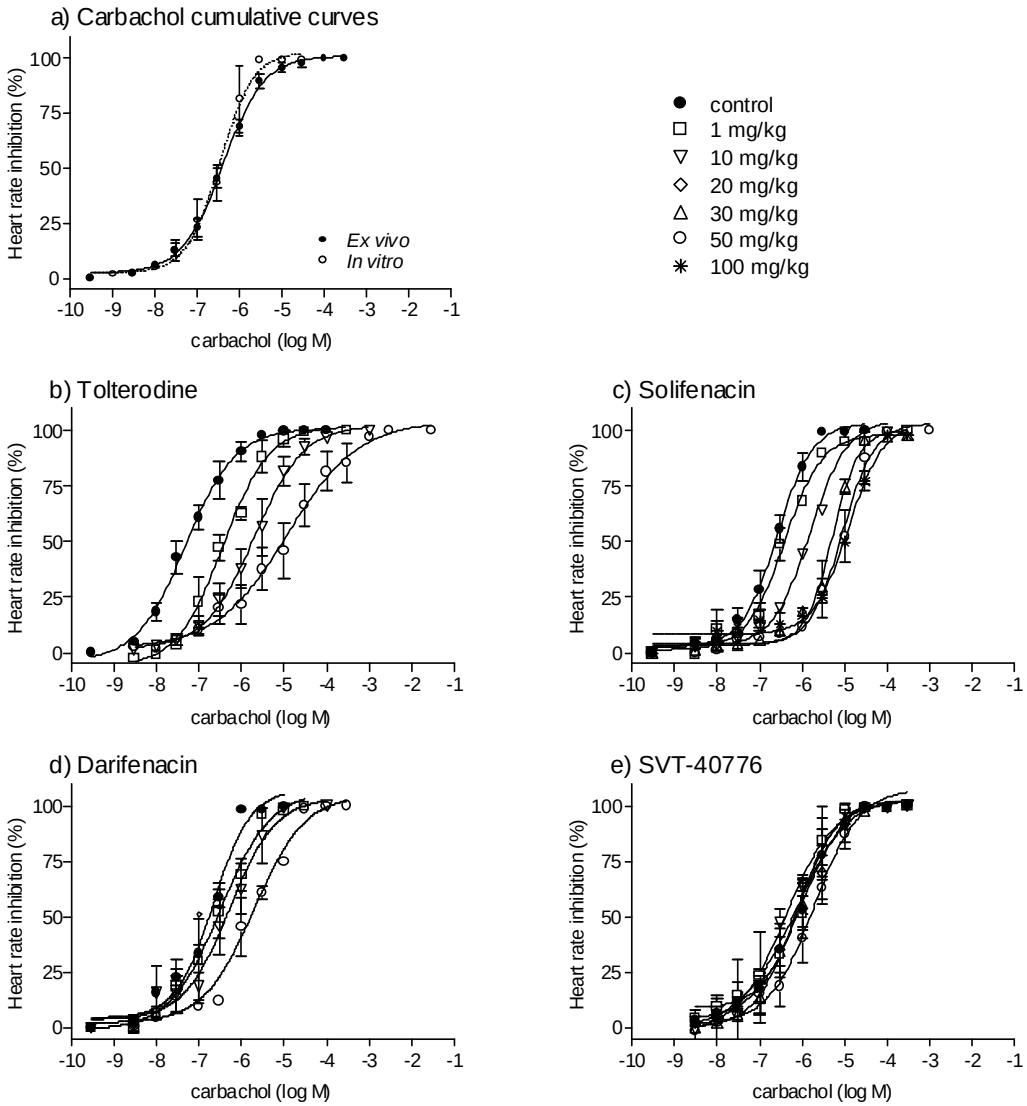


Figure 25. Effects of oral administration of muscarinic receptor antagonists on the concentration-response curves of CCh-induced contractions in mouse atria. Data are expressed as means ± SEM, n= 4-6 animals/dose.

Table 23a. Effect of oral SVT-40776 on CCh-induced heart contractions

COMPOUND	ATRIA pA_2-ED (CI) (mg/kg p.o.)	SLOPE
Tolterodine	0.15 (0.07-0.3)	0.92±0.09
Darifenacin	3.87 (2.1-7.5)	0.67±0.1
Solifenacin	2.05 (1.6-2.6)	0.96±0.05
SVT-40776	40.3 (32.6-51.6)	1.19±0.2

Table 24b. Oral bladder *vs.* atria ratios

COMPOUND	pA_2-ED (CI) (mg/kg p.o.)		SELECTIVITY RATIO (HA/BD)
	Bladder Detrusor (BD)	Heart Atria (HA)	
Tolterodine	0.66 (0.40-1.0)	0.15 (0.07-0.3)	0.21
Darifenacin	1.55 (0.35-6.71)	3.87 (2.1-7.5)	2.4
Solifenacin	1.39 (0.46-4.20)	2.05 (1.6-2.6)	1.5
SVT-40776	0.72 (0.41-1.10)	40.3 (32.6-51.6)	58

Footnote: Mice were sacrificed 3 h after receiving a single oral dose of antagonists or vehicle. Values are expressed as mean ± S.E.M.., n= 4-6 for each antagonist dose, 3-5 doses per compound

DISCUSION

Muscarinic antagonists are the mainstay in pharmacological therapy for the management of overactive bladder. They act by blocking the parasympathetic acetylcholine pathway and thus reducing the intensity of detrusor muscle contraction. However, none of the existing anticholinergic drugs are specific to the bladder, limiting their clinical efficacy and tolerability.

Many studies have shown that the M_3 receptor is the main receptor mediating contractile responses in normal bladder (Wang *et al.*, 1995; Hedge *et al.*, 1997; Chess-Williams *et al.*, 2001; Fetscher *et al*, 2002; Stevens *et al*, 2007). Pharmacological results have failed to demonstrate a role for M_2 muscarinic receptors in mediating direct contractions, concluding that this receptor plays either a small or a negligible role on that effect. Further studies using knock out mice have shown that the presence of M_3 is compulsory for any M_2 functional role (Matsui *et al*, 2000). It has been suggested that the relative contribution of M_2 receptors towards the overall direct contractile response becomes more important in diseased states (Pontari *et al*, 2004), although this finding has been recently been challenged (Stevens *et al*, 2007). Thus, the authors showed an increased sensitivity to CCh in both the neurogenic and idiopathic human overactive detrusors compared with the control human detrusor in tissue bath experiments by using CCh as the agonist. The M_2-selective antagonists (methoctramine, R0-320-6206) and M_1-selective antagonist (pirenzepine) had low affinities, whilst the M_3-selective antagonists (4-DAMP and darifenacin) had high affinities for the human detrusor muscarinic receptor in all three groups of tissues. The affinities (pK_B values) for the five antagonists were consistent with antagonisms at the M_3 receptor in all three groups; Schild plot analysis indicated an action at this single receptor subtype. Two conclusions were given by the author. The first one was that the contraction mediated by muscarinic receptors is enhanced in idiopathic and neurogenic overactive detrusors compared with control detrusor. The second is that the direct contractile response to CCh is mediated by the M_3 receptor in both human normal and overactive bladders, indicating no change in receptor subtype contribution to contraction in the disease state. Thus, nowadays the blockade of M_3 receptor is considered to be sufficient to obtain clinical efficacy.

Efficacy of antimuscarinic drugs for the treatment of overactive bladder has been evaluated in several clinical trials. A systematic review of 32 randomized controlled trials conducted by Herbison et al (2003) concluded that antimuscarinic agents

produced significant improvements in overactive bladder symptoms compared with placebo, even though the clinical relevance of these differences was uncertain. A recent update of a Cochrane systematic review has corroborated the efficacy of anticholinergic medication, also suggesting that improvements in symptoms may be associated with modest improvement in quality of life (Nabi *et al.*, 2006). The overall concept of improvement in quality of life was introduced to support a reported efficacy that did not reflect the real limited effectiveness, a handicap that none of lately available treatments has been able to overcome. From a rational point of view, a number of logical questions could arise: Is efficacy compromised because the dose level is limited? Is the dose limited because of the probability of adverse event occurrence? The incidence of typical muscarinic adverse events such as constipation or dry mouth has been shown to increase with dose (Chapple *et al.*, 2005; Hay-Smith *et al.*, 2005). But cardiac effects due to M_2 blockade, which would be unacceptable for a non-life-threatening condition, have been clearly underreported. One of the reasons for this fact could rely on the lack of interest of the clinical trial sponsors in supporting specifically-designed and carefully performed clinical studies to investigate such cardiac effects.

The potential benefits of muscarinic M_3 receptor selectivity in terms of tolerability and safety have been suggested (Andersson, 2002, Andersson *et al.*, 2007). In particular, we hypothesize that a highly selective drug for the M_3 *vs.* M_2 receptor subtype might be effective in treating overactive bladder, with a safer adverse-effect profile regarding cardiac effects. The cardiovascular safety of darifenacin and tolterodine has been evaluated in two clinical trials. The first one as part of a double-blind trial that assessed efficacy of therapy (Olshansky *et al.*, 2006; Romanzi *et al.*, 2005) The second one (Olshansky *et al.*, 2008) specifically focuses on evaluating heart rate variations during 24h post dosing using a Holter monitorization. Tolterodine significantly increased heart rate in comparison with darifenacin and placebo in both trials. In the first one, the proportion of patients with an increase in heart rate of ≥5 bpm from baseline to last observation was significantly greater with tolterodine (39.3%) than placebo (23.2%, $p<0.01$) or darifenacin (23.2%). The second one gave very similar results, pointing out that 4-5h post-dosing was the period of maximum heart rate increase for tolterodine. Trospium (FDA label information), oxybutynin (FDA label information) and propiverine (Dirschener *et al.*, 2000, Jabs *et al.*, 2001) have also shown to have effects on heart rate. Tiotropium, a well-known non-selective muscarinic antagonist developed for the treatment of chronic obstructive pulmonary disease (COPD), has showed to produce

cardiac effects and even an increase in deaths in clinical trials (*PADAC (FDA meeting 06 Sep 02). www.fda.gov/ohrms/dockets/ac/02/briefing).* Recently, a pooled clinical trial analysis of the safety of tiotropium reported that the risk of tachycardia was slightly elevated (Kesten *et al.*, 2005). Moreover, Barr et al (Barr *et al.*, 2006) have just published a meta-analysis of available randomized trials in which, among the adverse events reported, the authors point out that the frequency of arrhythmias was significantly higher with tiotropium than with placebo.

On the other hand, heart rate is also related to cardiovascular morbidity in the elderly (Andersson *et al.*, 2007). A prospective study involving 1311 men and women showed that there was 1.14 fold higher chance of developing new coronary events for each 5 bpm increment of heart rate (Aronow *et al.*, 1996) Non selective antimuscarinc generally decrease indicators of vagal cardiac tone. Thus, these drugs can increase resting heart rate and decrease heart rate changes after cessation of exercise, heart rate variability and baroreflex sensitivity (Andersson *et al.*, 2007). Whether or not the presently used non subtype selective agents at doses recommended for OAB treatment can produce changes of the vagal influence on the heart that might impose a risk for the patients has not been established. As the effects of antimuscarinics on heart rate are mediated by blockade of M_2 receptors, drugs that do not block these receptors would be preferable when treating patients with OAB and concomitant cardiovascular diseases (Andersson *et al.*, 2007).

Thus, taking into account all the above arguments, the overall objective of this study has been to show that SVT-40776 is a new kind of muscarinic receptor antagonist. Synthesized and patented by Farrerons *et al.* (2002), it exhibits a differential pharmacological profile suggesting a clinical potential to treat OAB by avoiding any cardiac effect associated with the antagonism of M_2 receptors.

Our first step has been to establish the affinity and selectivity to human muscarinic receptors of SVT-40776 (objective 1). Secondly, to assess that the potency and the selectivity of the compound is maintained by using both in vitro and ex vivo isolated bladder and atria and whole animal studies, thus showing that the *in vitro* profile can be reproduced in the functional activity (objective 2 to 4).

Characterization of SVT-40776 binding affinity and selectivity in the muscarinic receptor family and evaluation of the binding characteristics to the M_3 receptor

The affinity and selectivity of SVT-40776 for the human M_3 mACh receptor subtype was determined by [^{3}H]NMS receptor binding competition in CHO cell membranes containing muscarinic receptors from NHI and in CHO cell membranes expressing human muscarinic receptors from Receptor Biology Inc.. SVT-40776 showed the highest affinity (K_i= 0.19 nM) and selectivity (203-fold *vs.* M_2) for the human M_3 receptor subtype, in comparison with other muscarinic antagonists. The estimated affinity and selectivity of oxybutynin, tolterodine, darifenacin and solifenacin are compatible with previously published data (Hegde , 2006). SVT-40776 exhibited higher affinity for the human M_3 receptor than for the rest of muscarinic receptor subtypes.

The system using CHO cells transfected with the human receptors was tested for selectivity using methoctramine and 4-DAMP, described as M_2 selective and M_3 selective antagonists respectively. Methoctramine showed a Ki of 98 nM on the M_2 receptor, and did show affinities for M_1 and M_3 of 260 and 3321 nM respectively. 4-DAMP, on the other hand, did show a moderate M_3 selectivity, with a Ki of 0.8 nM on the M_3 receptor vs 10.9 nM and 2.4 nM on the M_1 and M_2 respectively. Darifenacin showed marked M_3 *vs.* M_2 selectivity (55-fold), while oxybutynin (2.9-fold) and solifenacin (8.6-fold) showed less selectivity for the M_3 receptor and tolterodine completely lacked selectivity (0.4-fold). When using CHO membranes from Receptor Biology Inc., the competitors showed similar ratios. Darifenacin showed M_3 *vs.* M_2 selectivity (29.4-fold), while oxybutynin (5.7-fold) and solifenacin (5.9-fold) showed again lower selectivity for the M_3 receptor. Tolterodine completely lacked selectivity (0.5-fold). Our results are compatible with previously published data for the known compounds (Hegde, 2006) thus validating the assay and reinforcing the results obtained with SVT-40776.

Another important feature found for SVT-40776 was the reversibility of its antagonism. To elucidate the type of interaction of SVT-40776 with human M_3 and M_2 receptors, [^{3}H]NMS saturation curves were performed in the presence of different antagonist concentrations. The presence of SVT-40776, darifenacin, solifenacin and tolterodine produced concentration-dependent rightward shifts of [^{3}H]NMS saturation curves in M_3 receptor when compared to that obtained in the absence of antagonists These

displacements were accompanied by a concomitant increase in the K_d value without significantly changing the B_{max} value in the case of SVT-40776, darifenacin, solifenacin and tolterodine for the M_3 receptor, which was compatible with a competitive binding. As expected, 4-DAMP mustard induced a concentration-dependent reduction in the B_{max} value without modifying the K_d parameter, thus confirming its irreversible profile. Additionally, [^{3}H]NMS saturation studies were carried out with CHO-M_2 cell membranes preparations to further examine SVT-40776 binding features. SVT-40776 displaced [^{3}H]NMS from the binding site, increasing its K_d without any change in B_{max} value. Such results indicate that the compound also behaved as a competitive antagonist of the M_2 cholinergic receptor.

This data is coincident with further experiments carried out in our laboratory and presented in Annex I (paper in press) using [^{3}H]SVT-40776. In these, the affinity (expressed in terms of K_d value) of [^{3}H]SVT-40776 for M_3 (K_d= 0.54 nM) also correlated with the K_i value (K_i= 0.19 nM) obtained in the [^{3}H]NMS binding experiments, indicating that the compound binds specifically and selectively to this receptor. The K_d obtained for M_1, M_4 and M_5 (in all cases, higher that 1 nM) were also in agreement with the affinities found in the [^{3}H]NMS competition assays. On the other hand, no specific binding to the M_2 receptor was detected using [^{3}H]SVT-40776, even when the presence of the receptor in the cells had been previously confirmed with [^{3}H]NMS. The existence of an allosteric site in the human M_2 receptor partially overlapping the corresponding orthosteric site has been hypothesised (Jakubik *et al.*, 2000; Krejcí *et al.*, 2004, Christopoulos *et al.*, 1998). One possible explanation could be that SVT-40776 shows affinity for such allosteric site only after the conformational change induced by the presence of a ligand (agonist or antagonist) in the orthosteric site.

Thus, as a general conclusion from this group of binding studies, SVT-40776 appears to be the most potent and selective M_3 *vs.* M_2 receptor antagonist identified to date for OAB treatment. Furthermore, SVT-40776 behaves as a reversible competitive antagonist, which predicts a good tolerability profile regarding M_2 related adverse effects.

Determination of SVT-40776 functional activity in mice bladder and mice heart

In vitro functional studies performed in isolated tissues demonstrated that SVT-40776 was the most potent compound inhibiting CCh contractions in mice bladder smooth

muscle. SVT-40776 showed the ability to induce a rightward parallel shift on the cumulative agonist concentration-response curves, obtaining a pA_2 of 9.5. Antagonist activities of tolterodine, solifenacin and darifenacin were 8.4, 8.6 and 8.7, respectively. Methocratmine, pirenzepine 4-DAMP and 4-DAMP mustard were also tested in our system. The pA_2 obtained was 6.3, 6.5, 9.6 and 9.1 respectively. These findings suggest the exclusive involvement of M_3 muscarinic receptor in the direct contractile response to CCh, as all the antagonists present a pA_2 compatible with its M_3 affinity (Ki). Interestingly, 4-DAMP mustard, as described, showed an unsurmountable profile. 4-DAMP mustard produces a non parallel righward displacement on the cumulative curve of CCh and decreases significantly the $E_{max.}$ 4-DAMP mustard has been described as an irreversible antagonist (Ehlert, 1996). The irreversible antagonists could give dose-response curves very similar to the non-competitive antagonists, a decrease on the E_{max}, but contrary to the non-competitive antagonists, presenting a non parallel displacement of the dose response curve. Also, non competitive antagonists can produce shifts to the right of dose-response curves with no diminution of maximum response or shifts with diminution of E_{max} (Kenakin T, 1996). On the other hand, the elevated affinity was not completely reflected in the pA_2 value (lower than the competitive antagonist 4-DAMP). This underestimation is due to the method used to calculate the pA_2, the Schild plot, as indicated for competitive reverible antagonists (Richard *et al.*, 2003) . In these cases, the calulations can be accepted but named apparent pA_2 (Fetscher *et al*, 2002) .

Thus, while tolterodine and solifenacin did not significantly alter maximum CCh response, darifenacin reduced E_{max} in a dose-dependent fashion, which is compatible with an unsurmountable behaviour. These results are consistent with previous findings in the rat (Hegde *et al*, 1997), in the dog (Choppin *et al*,, 2001), in the mouse (Yamada *et al*,, 2006) and in the human bladder (Fetscher *et al*,, 2002) . It has been described as reversible but a non competitive antagonist. The slope in this case is superior to unit, which also reinforces this feature. Nevertheless, the incubation with SVT-40776 at concentrations equal or higher than 10 nM induced minor non-dose-dependent decreases in the E_{max}. The pharmacological meaning of these results has not been elucidated so far and would require further investigation. These concentrations are between 25 and 250-fold higher than the intrinsic activity of SVT-40776. Therefore, taking into account the E_{max} effect, an unsurmountable effect at very high concentrations cannot be ruled out. In contrast, the behaviour at concentrations within its affinity for the M_3 receptor is compatible with a competitive antagonist as well as

tolterodine and solifenacin but conversely to darifenacin. One explanation could be, taking into account that SVT-40776 and darifenacin had slopes greater than 1, that there was an incomplete equilibration or a depletion of the antagonist from the medium as a consequence either of binding to receptors or other structures. Equilibration times did not exceed 60 min. Experiments with different incubation times with a high concentration were carried out for SVT-40776 (15 min, 30 min, 45 min and 60 min). The responses were similar in all cases, instead of expecting a minor Emax affectation at shorter incubation times, if there had been a problem of incomplete equilibration or binding to other structures. Thus, at 15 min, SVT-40776 was already decreasing the E_{max}. Another explanation would be if SVT-40776 behavied as a non-competitive antagonist. A non- competitive antagonism should have shown a competitive dose-response in the short incubation time but a reduced maximal response (E_{max}) at larger incubation periods (Kenakin T, 1996) . Neither was this the case. Thus, further work should be carried out to clarify this point. Referring to darifenacin, it is worth pointing out that it did behave as a reversible competitive antagonist in the binding studies but showed an unsurmountable behaviour in the isolated tissue studies and this profile is compatible with the published data (Yamada *et al*, 2006). One hypothesis could be the different pharmacological nature of the compounds used to compete with darifenacin. (using an antagonist (NMS) in the former and an agonist (CCh) in the latter). The antagonist NMS has to compete most probably for the same binding site. However, the agonist does not necessarily have to share the same binding site as the tested antagonist and it would be compatible with the the proile of a reversible but a non competitive antagonist to CCh.

In atria preparations, CCh curves were surmountably antagonized by all the compounds evaluated in a concentration-dependent fashion, with parallel rightward displacements, at lower potencies than in the bladder. The rank order of antagonist activities (pA_2) was tolterodine (8.5), solifenacin (7.8), darifenacin (7.3) and SVT-40776 (7.3). It should be pointed out that SVT-40776 did not show any relevant affinity at the M_2 receptor subtype in comparison with tolterodine, solifenacin and darifenacin. In fact, SVT-40776 exhibited the highest bladder *vs.* atria selectivity (199-fold), therefore predicting a safer cardiovascular profile than the other antagonists tested. All slopes in this case were similar for the 4 compounds tested and between 1.1-1.2.

The *ex vivo* isolated tissue functional studies were intended to obtain a closer approach to the *in vivo* situation, as they integrate the pharmacokinetic (PK) and distribution

pattern of the compounds. We studied previously the PK profile in mice for SVT-40776 and all the standards in order to ensure that there were relevant plasma and tissue levels for all the compounds at the time points studied. All the compounds showed good oral absorption and plasma levels during several hours post-dosing. These data are not presented as they are not the objective of this thesis. The maximum plasma levels were obtained between 1h-3h post dosing for all the compounds. Consequently, 3h was the timepoint chosen to further evaluate the *ex vivo* selectivity. Furthermore, an *ex vivo* time course was carried for all the compounds in the bladder.

Our first concern was to test if the stress induced to the animal due to the fasting conditions and the dosing manipulation would interfere with the CCh response. We showed that tissue preparations coming from fasted and handled mice do have similar responses compared to the direct *in vitro* studies. Thus, the pEC_{50} obtained for CCh in the the detrsuor *in vitro* or *ex vivo* protocol was 5.66 (5.58-5.74) and 5.19 (5.13-5.25) respectively. Our second concern was related with the technical manipulations carried out on the tissues: Dissection, preparation of strips and tissue warming, as all these procedures could contribute to an accelerated release of the drug from the tissue. However, clear dose responses were obtained for all the antagonists and the intra-animal differences were minimal and consistent. Finally, darifenacin showed the same unsurmountable profile in the *in vitro* and *ex vivo* protocol confirming the non effect on the manipulation of the strips versus the compound binding to the tissue. The response to KCl was also similar in all the tissues. Thus, taking into account all these points, we claim that the technique is reliable for study purposes. In Table 25 control CCh pEC50 values from detrusor and atria preparations can be seen, comparing *in vitro* and *ex vivo* experiments. These values are very similar, which demonstrates the reliability of the *ex vivo* technique.

Table 25. Comparative *in vitro* and *ex vivo* control CCh pEC_{50} obtained in detrusor and in atria experiments.

pEC_{50} (CI)	**Mice** ***In vitro*** **(n)**	**Mice** ***Ex vivo*** **(n)**	**Dog** ***Ex vivo*** **(n)**
Detrusor	5.66 (5.58-5.74) (4)	5.19 (5.13-5.25) (71)	5.82 (5.89.-5.74) (4)
Atria	6.23 (6.13-6.33) (10)	6.55 (6.37-6.73) (27)	NT

NT = not tested

SVT-40776 inhibited CCh-induced bladder contractions in a concentration-dependent manner after its oral administration in rodents. Neither tolterodine nor solifenacin nor SVT-40776 significantly altered the maximum response to CCh. On the contrary, darifenacin significantly reduced the E_{max} at 50 mg/kg, verifying the unsurmountable antagonist profile shown in the *in vitro* protocol. Differentially to darifenacin, SVT-40776 did not show an unsurmountable profile in this case, thus reinforcing its competitive profile. Both compounds, SVT-40776 and darifenacin, despite showing a different profile of the rightward dose-response curves, presented a very similar slope (1.4 vs. 1.8, respectively) and also similar to the *in vitro* assays. Likewise, darifenacin exhibited less potency than tolterodine and solifenacin in the atria*,* which accounts for the M_3 *vs*. M_2 receptor selectivity attributed to darifenacin (Gillberg *et al*,, 1998) and also shown in the previously described binding experiments. Besides, the pharmacological selectivity of SVT-40776, stated as the ratio between muscarinic cardiac effects *vs.* bladder effects, was 58-fold, which compared favourably with tolterodine (0.21-fold), solifenacin (1.5-fold) and darifenacin (2.4-fold). Interestingly, in atria SVT-40776 did not induce any significant rightward shifts of the CCh curves at doses up to 30 mg/kg. Since the *ex vivo* model reflects the physiological conditions more accurately, these data support that SVT-40776 is the first M_3 antagonist devoid of any M_2 functional affinity.

Determination of SVT-40776 time course of the therapeutic effect in mice and dog bladder

When a time course was carried out in the bladder *ex vivo* protocol, clear time-dependant responses were observed for all the compounds that most probably correlate with their pharmacokinetic and tissue distribution in mice. The CCh pEC50 was similar at all four time points tested. SVT-40776 showed efficacy from 1h post – dosing up to 24h. Thus, SVT-40776 showed that it was rapidly distributed to the target organ, as well as to other organs such as heart (see *ex vivo* atria paragraph), and it remained in the bladder for a very long period (24h). Comparatively, darifenacin did not produce any inhibition untill 3h post-dosing. At 1h there was a complete lack of activity. At 5h post-dosing, darifenacin was the most potent antagonist. Taking into account that tolterodine is highly metabolised in mice (Appell, 1997) and that the metabolite is active (see Chapter 1.2.), the activity observed at 3h is suspected to be due to the parental compound plus the metabolite. This does not occur with SVT-40776, which metabolises to one major non active metabolite (data not shown).

The experiments in dogs have to be considered preliminary as sample sizes used were reduced due to ethical reasons. Nonetheless, the results were similar to those in mice. We used five animals for the whole experiment. Four bladder samples were taken from each animal with a two weeks washout period between operations. The atria was not tested in this *ex vivo* protocol as it would have implied using one animal per time point and dose, unacceptable from an ethical point of view. Also, due to the number of animals required for defining one compound, only SVT-40776 was tested in these conditions. However, the heart rate was deeply evaluated *in vivo* in this species in different experimental approaches following ICHs guidelines and no effects up to 10 mg/kg were observed (SALVAT; data on file).

Detrusor smooth muscle from control animals 1h and 3 h after receiving an oral dose of vehicle had no significantly different behaviour compared with non-treated animals. CCh induced concentration-response curves yielding pEC50 = 5.82 (5.89-5.74). CCh responses were comparable with those in mice both in the *in vitro* (5.66 (5.58-5.74)) and the *ex vivo* protocol (pEC50 = 5.19 (5.13-5.25)). Right shifting of the CCh response curves to SVT-40776 were also obtained. They were comparable to those in mice and yielded a Schild regression line close to unit. SVT-40776 was able to inhibit dog detrusor CCh-induced contractions after oral administration, by being more potent after 3h post-dosing than after 1h. Thus, the pA-ED_{50} at 1h and 3h was 1.09 mg/kg and 0.31 mg/kg respectively. If the 3h time point is compared between species, 0.72 mg/kg and 0.31 mg/kg was obtained in mice and dog respectively. These results showed that SVT-40776 was absorbed and clearly distributed on the bladder 1h after oral dosing producing a clear blockade of the CCh –induced contraction in the muscle. This effect increased 3h post-dosing suggesting a prolonged duration of action of the compound on urinary bladder. It was very relevant to find such effect in dogs as well as in mice as it would better support the effectiveness of the compound by using two different species. Rodents and dogs are the two types of animals recommended by ICH guidelines for carrying out the drug development of a new chemical entity, including safety and toxicology studies. Moreover, this is the first time that an isolated detrusor *ex vivo* protocol has been described (Salcedo *et al*,, 2002).

Determination of the *in vivo* fuctional profile on the urodynamia of anaesthetised guinea pig of SVT-40776

To further confirm the above observations, it was necessary to test the efficacy of the compound in a more complex pharmacological model based on whole anesthetized animal. Guinea pig was used and it was the selected species for two reasons: primarily M_3:M_2 receptor population proportion (1:3) was similar to human (1:3) compared to rat (1:9) (Fetscher *et al*., 2002) and secondly the similarity of the urethra morphology and functionality to human's urethra (Walters *et al*., 2006).

As a general conclusion, SVT-40776 did inhibit micturition contractions in the two models used, the isovolumetric and the cystometric model, without affecting the arterial pressure (MAP) and/or the heart rate (HR).

Referring to the isovolumetric model, SVT-40776 inhibited isovolumetric-induced contractions in a dose-dependent fashion, without affecting any cardiovascular parameter. This model has been widely used for testing compounds directed to OAB (McMuray *et al*, 2006; Modiri *et al*,, 2002) and examines the behaviour of the urinary bladder after acute and complete ligation or blockade of the bladder neck. This blockade of the emptying of the bladder creates the isovolumetric system. After filling the bladder up to a point where a micturition event occurs, the filling is stopped and the bladder continues to generate rhythmic isovolumetric contractions of similar amplitude, frequency and duration. Effects on these contractions are very easily and consistently observed (Birder *et al*,, 1993; Yoshiyama *et al*,, 1993). As it is a non-physiological model, extrapolation of drugs effects to the normal bladder condition must be carried out with caution. However, a number of studies have reported clear effects with drugs also able to alter those key parameters measured during standard human cystometry (Modiri *et al*,, 2002; McMurray *et al*,, 2006; Yokoyama *et al*,, 2007; Palea *et al*,, 2007), suggesting a good correlation between both situations. Thus, SVT-40776, oxybutynin, tolterodine, darifenacin and solifenacin inhibited bladder contraction amplitude in a dose-dependent manner. Nonetheless, SVT-40776 proved to be the most potent muscarinic antagonist in this *in vivo* model, with an ED_{25} of 6.9 µg/kg. MAP and HR were calculated simultaneously at each single tested dose for every compound. SVT-40776 never produced an effect on MAP or HR up to the highest dose tested of 1000 µg/kg. However, tolterodine showed an ED_{25} of 97.6 µg/kg and induced hypertension at 267 µg/kg. When compared at the equipotent dose of 1 µg/kg for SVT-40776 and 3

µg/kg for tolterodine, SVT-40776 did not produce any increase on the cardiovascular parameters. On the contrary, tolterodine increased the baseline HR by 31%.

These data provide *in vivo* functional evidences of the potential advantages of a drug selective for the M_3 receptor *vs.* M_2 regarding cardiovascular safety.

Regarding cystometry, this model is the most commonly utilised for exploring bladder function (Doi *et al*,, 1999; Pandita *et al*,, 2000; Testa *et al*,, 2001; Gu *et al*,, 2002). It is based on a slow filling of the bladder while measuring intravesical pressure, via a bladder dome catheter, until the point of fullness in order to elicit a micturition response. The same procedure is carried out in humans to diagnose detrusor overactivity and the OAB severity. Similar parameters are measured in the human and animal cystometry, thus, the MP, the BC , the TP and the RP (residual volume for human).

All compounds tended to increase, with more or less potency, the TP and the BC. MP was also increased or not affected in this model. This point could be controversial as this result differs from the isovolumetric model, where MP contraction is always inhibited by M_3 antagonists (Ishizuka *et al*,, 2002; Angelico *et al*,, 2005, Ohtake *et al*,, 2008). In that model any tested compound inhibited the MP apart of oxybutynin that increased it. Some authors have shown a decrease in MP for tolterodine (Ishizuka *et al*,, 2002; Angelico *et al*,, 2005) and solifenacin (Ohtake *et al*,, 2008). Possible explanations could be the use of a different animal species. These authors used rat instead of guinea pig. However, guinea pig seems to be a better option than rat to reproduce the human urodynamia, as explained previously. When the same cystometric model is used in conscious animals, the MP does not seem to correspond to the same physiological process as when the animal is anaesthetised (Angelico *et al*, 2005). Also, in the isovolumetric model, the repetitive contractions observed are different from the ones seen in the cystometry, and closer to those observed in conscious animals. Thus, cystometric model in anesthetised animals produces a micturition process that induces a complete voiding of the bladder. On the other hand, in the isovolumentric model , the contractions observed are not efficient contractions, so do not fully void the bladder, as in the conscious model, where the contractions are neither effective (Angelico *et al*,, 2005). Thus, in some way the isovolumetric model is more similar to the conscious model in animals, and the cystometric model is more similar to human cystometry.

Thus, it is reasonable to think that the same compound may have two behaviours as different processes have to be modulated, depending on the model, with the participation of different micturition reflex players. So, we should analyse the results from each model separately.

On the other hand, TP and BC are direct measurements of the storage phase. Antimuscarinics are known to be acting on this phase (Abrams *et al*., 2005). Thus, an increase of these two parameters represents a promising result for a putative treatment for OAB, although these animals are non pathological animals. Consequently, to better understanding this effect, the use of OAB animals would be advisable. However, the pathological models are far from optimal as most use acetic acid (AA) to induce bladder irritation to produce an irregular bladder contraction compatible with overactive bladder detrusor hiperactivity. This AA model is more suitable for testing drugs that modulate afferents acting on the detrusor muscle. Antimuscarinics act mainly on detrusor muscle directly. So, all results obtained here should be treated with caution. Therefore, increasing TP, so prolonging the time and the pressure when micturition should start is the main efficacy parameter as it is a direct effect of blocking the muscarinic receptors in the bladder. BC is directly related with TP. We have always observed that both activities changed in the same direction although the magnitude of the response does not perfectly correlate, i.e. tolterodine. This effect on TP and BC has been observed in all the antimuscarinics tested. In the case of solifenacin and oxybutynin it was only significant for BC at all doses tested. SVT-40776 was the most potent compound compared with the other antimuscarinics in the *in vitro* and *ex vivo* experiments and the results obtained in this model showed the same profile. SVT-40776 was statistically significant from the dose of 0.6 mg/kg, compared to tolterodine that showed efficacy at 3 mg/kg, solifenacin and oxybutynin that, although not statistically significant, did show a tendency both at 3 mg/kg.

RP is an indirect measurement of residual volume. When micturition pressure does not completely void the bladder, some urine remains contributing to maintaining a high intravesical pressure. RP gives the intravesical pressure after micturition. In non-treated animals RP should be around zero. In our system, there is a remaining intravesical pressure of about 5 cm H_2O in each micturition cycle. It is important to point out that there is no accumulation of saline in the bladder after the five micturitions as the RP maintains the same value throughout the whole procedure. When a high RP is

observed, it can be suggested that all mechanisms involved in bladder emptying have been blocked. As antimuscarinics are the drugs being tested in this study, probably the cause for this RP at higher doses is an almost complete blockade of all muscarinic receptors in the detrusor allowing the physiological ACh to act only partially after the initiation of micturition. Most interestingly, SVT-40776 is the only compound that shows a window between the effect on TP and the effect on RP. At 1 mg/kg the effect on TP is highly significant but no effect is seen on the RP. However, tolterodine does not present any window. In fact, tolterodine does not have significant effects on TP when affecting the RP. Oxybutynin has an even more remarkable effect. It (1 mg/kg) only shows a slight tendency to increase TP when significantly increasing RP. It is tempting to speculate that a selective M_3 *vs.* M_2 compound exhibits a wider window in this model. Alternatively, it could be explained due to an extra non muscarinic activity that has been described for oxybutynin. The mechanism of action of oxybutynin is attributable to its antimuscarinic activity combined with relaxant and local anaesthesic activities on bladder smooth muscle. The direct relaxant activity is not mediated by the inhibition of tissue phosphodiesterases but probably reflects oxybutynin's local anaesthesic properties and associated effects on Ca^{2+} fluxes (Tiwari, 2006). Thus, together with its non-selective antimuscarinic activity, oxybutynin would be strongly relaxing the detrusor producing the observed effect. MAP was also evaluated. Tolterodine and solifenacin increased MAP very significantly. On the contrary, oxybutynin did not produce such effect, probably due to the same reason given previously to explain the RP increase. SVT-40776 increases MAP but less extensively than solifenacin and tolterodine. When analysing together the whole cystometric profile the compounds behave following the receptor affinity for the M_3 receptor (see Table 26). Thus, for binding affinities the order (more to less affinity) is SVT-40776>darifenacin >tolterodine>solifenacin. For the functional in vivo potency the order is SVT-40776>darifenacin > solifenacin> tolterodine.

Table 26. Comparative binding affitinty *vs. in vivo* efficacy

COMPOUND	BINDING AFFINITIES (Ki values, nM)	BLADDER PRESSURE (BP) INHIBITION ED_{25} (µg/Kg)
Tolterodine	4.13 ± 1.7	97.55
Darifenacin	3.05 ± 0.2	22.69
Solifenacin	7.3 ± 1.4	72.82
SVT-40776	0.19 ± 0.09	6.97

On the other hand, as previously mentioned, the results obtained here cannot be directly correlated in absolute terms with those from the isovolumetric assay. In the cystometric model all compounds present activity at doses about 100-fold lower than in the isovolumetric model. However, the ratio selectivities and the relative potencies between compounds are maintained (see Table 27).

Table 27. Summary of selectivity ratios for the *in vitro*, *ex vivo* and *in vivo* assays

COMPOUND	Binding affinity	Functional *in vitro*	Functional *ex vivo*	Functional *in vivo* (Isovolumetric model)
Tolterodine	0.5	0.8	0.21	2.7
Darifenacin	2.9	31.6	2.4	22.0
Solifenacin	5.9	6.3	1.5	13.7
SVT-40776	203.7	199	58	143.5

The main objective of this study was to characterise the compound from the M_3 *vs.* M_2 point of view. However, we believe it is important to summarily describe the putative effects of SVT-40776 referring to the other three muscarinic receptors. All muscarinic antagonists tested differed in their binding affinity for the five human recombinant muscarinic receptors (Table 8). While tolterodine did not discriminate between the five subtypes, oxybutynin showed marginal selectivity for M_3 over M_2/M_5 subtypes and did not discriminate between M_3 and M_1/M_4 subtypes; solifenacin showed marginal selectivity between M_3 and M_2 subtype and did not discriminate between M_3 and M_1/M_4/M_5 subtypes; darifenacin had a high degree of selectivity for M_3 over M_1/M_2 subtypes and modest selectivity for M_3 over M_4/M_5 subtypes. Differently, SVT-40776 showed high degree of selectivity for M_3 over M_2 subtype and marginal selectivity for M_3 over M_1/M_4/M_5 subtypes (Table 8).

Due to its high M_3 affinity, it could be expected that SVT-40776 could block M_3 receptors in glands and other (non-bladder) smooth muscles rich in this receptor

subtype. The salivary glands are known to contain M_1 and M_3 receptors, whose activation is crucial for salivary secretion (Culp *et al.* 1996). Studies carried out by Gautam et al. using M_1/M_3 receptor double-KO mice, showed that pilocarpine-induced salivary secretion was abolished in those mice (Gautam *et al.* 2004), which is consistent with the concept that muscarinic receptor mediated stimulation of salivary secretion is mediated by a mixture of M_1 and M_3 receptors. In order to assess a possible dry mouth side effect produced by SVT-40776, we performed studies in our lab comparing inositol phosphate accumulation in response to muscarinic receptor activation in mice and human bladder and salivary glands. SVT-40776 exhibited a high selectivity for bladder over salivary gland (23-fold in mice tissues and 14-fold in human samples) (Balsa *et al*. 2005, Enrich *et* al. 2008, paper in preparation).

In addition to present a good uroselectivity, an antimuscarinic drug developed to treat OAB and which will be addressed mainly to aged patients, should avoid CNS effects. Although all five muscarinic receptor subtypes are expressed in the brain, M_1 receptors play a major role in higher cognitive processes such as learning and memory (Caccamo *et al.* 2006). Thus, antagonism of M_1 receptor by antimuscarinics could produce important undesirable CNS side effects. Taking into account the moderate affinity showed by SVT-40776 on M_1 receptor subtype, we investigated the drug penetration into the brain, in dog and mouse after oral administration of [^{14}C]SVT-40776 (data not shown). The obtained results showed very low levels of radioactivity in dog and mouse brain (17-fold lower than in urinary bladder) suggesting a low penetration of SVT-40776 through the blood-brain.barrier (Lagunas *et al.* 2005).

Although it is accepted the role of M_2 and M_3 receptors located in the detrusor muscle on bladder contraction, recent studies have demonstrated their presence on the human bladder mucosa/urothelium (Mansfield *et al*. 2007). Molecular reverse transcription (RT)-PCR studies have shown the expression of mRNA for M_2 and M_3 receptors in detrusor and mucosa from control patients as well as in detrusor and mucosa of patients with refractory idiopathic detrusor overactivity. The studies showed a significant less expression of M_3 receptor mRNA in the mucosa but not in the detrusor of overactive bladder (Mansfield *et al.* 2007). Although the precise role of muscarinic receptors in the bladder mucosa it is not clear yet, it has been suggested that their stimulation could release an unknown urothelial-derived inhibitory factor (UDIF) able to modulate the detrusor contractile activity (Hawthorn *et al.* 2000, Scott *et al.* 2005, Mansfield *et al.* 2007, Murakami *et al*. 2007). Urothelial muscarinic M_2 receptors could

mediate UDIF release (Scott *et al*. 2005). It can be thought that this inhibitory factor could be decreased in the overactive bladder, causing at least in part, an increase in bladder contraction. In that case, an antagonistic activity on M_2 receptor would decrease efficacy for an antimuscarinic agent in the treatment of overactive bladder. On the contrary, SVT-40776, lacking activity on M_2 receptor, would have an advantage in the treatment of the syndrome.

Thus, as a general conclusion, the present study has shown that SVT-40776, a novel substituted quinuclidine derivative, is the most potent and selective muscarinic receptor antagonist described so far for the treatment of overactive bladder. Its functional selectivity for urinary bladder over cardiac tissues is in the order of 100-fold, a window not reached with any of the current treatments. This wide experimental safety window can provide SVT-40776 with an advantage in the treatment of this condition. The compound has successfully completed Phase I clinical trials and is currently undergoing Phase II clinical trials for the treatment of overactive bladder.

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LIST OF ABBREVIATIONS

Ach	= acetylcholine
ATP	= adenosine triphosphate
Bpm	= beats per minute
CCh	= Carbachol
B_{max}	= maximun binding
BC	= bladder capacity
DRG	= sacral dorsal root ganglia
4-DAMP	= 4-diphenylacetoxy-N-methylpiperidine methiodide
EUS	= external urinary sphincters
EC_{50}	=concentration of the agonist necessary to produce a 50% efficacy.
E_{max}	= maximum effect
HR	= heart rate
HPMC	= hydroxypropilmethylcellulose
5-HT	= 5-hidroxytryptamine (serotonin)
ICS	= International Continence Society
IC_{50}	= concentration to produce 50% of the required effect
ICS	= International Continence Society
IMG	= the inferior mesenteric ganglion (IMG)
K	= elimination constant
K_d	= dissociation constant
Ki	= inhibition constant
UI	= urinary incontinence
OAB	= overactive bladder
MAP	= mean arterial pressure
MP	= micturition pressure
nH	= Hill coefficient
NMS	= N-methyl-scopolamine
pA_2	=concentration of the antagonist necessary to produce a 2-fold displacement of the EC_{50}
pEC_{50}	= - log EC50
PAG	= periaqueductal gray
PBS	= phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4).
PG	= prostaglandins

PK = pharmacokinetics

PMC = Pontine Micturition Centre

$P2X_3$ = subtype of purinergic receptors

SNP = sacral parasympathetic nucleus

NGF = nerve grouw factor

NO = nitric oxide

TP = threshold pressure

TRP = transient receptor potential

tkrA = takikinin A receptor

tkr B = takikinin B receptor

$TRPV_1$ = transient receptor potential vanilloid 1

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ANNEX I

PAPER IN PRESS **; Enviat a British Journal of Phamacology el 22 Setembre 2008**

Title page

Binding properties of SVT-40776, a highly selective M_3 over M_2 muscarinic receptor antagonist developed for the treatment of overactive bladder

Summary

Background and purpose. M_3 muscarinic receptors play a major role in mediating direct contraction of the bladder. Although antimuscarinics are commonly used for the treatment of overactive bladder, their lack of selectivity leads to classical antimuscarinic side effects. This study reports the binding characterization of a novel quinuclidine derivative, SVT-40776, as a M_3 selective antagonist.

Experimental approach. Saturation binding assays with [^{3}H]NMS and [^{3}H]SVT-40776, competition studies with [^{3}H]NMS and association-dissociation kinetic experiments with [^{3}H]SVT-40776, were employed to study the binding properties of SVT-40776. The functional behaviour at the human M_3 receptor was characterized by measuring intracellular Ca^{2+} mobilization in CHO-M_3 cells.

Key results. SVT-40776 exhibited a high affinity for the human M_3 receptor (0.19 nM) where it behaved as a competitive reversible antagonist. SVT-40776 also showed a high degree of selectivity for M_3 over M_2 receptor subtype (203-fold). In addition, SVT-40776 inhibited acetylcholine-induced Ca^{2+} mobilization through human M_3 receptors in a concentration-dependent fashion (K_B= 0.28 nM), being 7-fold, 7-fold and 4-fold more potent compared to the antimuscarinic agents: tolterodine, darifenacin and solifenacin, respectively.

Conclusions and implications. The quinuclidine derivative SVT-40776 is a novel M_3 muscarinic receptor antagonist with high affinity for the human M_3 receptor. The compound possesses a higher degree of selectivity for M_3 over M_2 receptor subtype, when compared to the marketed antimuscarinic drugs. The binding properties of SVT-40776 suggest that this novel compound could render improved efficacy and a better tolerability over currently available treatments for overactive bladder.

Keywords: Antimuscarinic, M_3 receptor, urinary bladder, OAB, SVT-40776, oxybutynin, tolterodine, darifenacin, solifenacin, carbachol

Abbreviations: mACh, muscarinic acetylcholine; CCh, carbachol; IP_3, inositol 1,4,5-trisphosphate; [^{3}H]NMS, [N-methyl-^{3}H]scopolamine methyl chloride; GPCR, G protein-coupled receptor; OAB, overactive bladder; CHO, Chinese hamster ovary;

Introduction

Overactive bladder is defined by the International Continence Society (ICS) as a condition characterized by urgency with or without urge incontinence, usually with frequency and nocturia (Abrams et al., 2003). Results of the EPIC study conducted in Western Europe and Canada showed that the overall prevalence of overactive bladder symptoms in the general population was 11.8%. Similar prevalence was found between men and women and increased with age (Irwin et al., 2006a). Few population-based surveys of overactive bladder symptoms have been published, but all agree that patients' quality of life is severely affected (Irwin et al., 2006b). The symptoms of overactive bladder are thought to result from involuntary bladder contractions during the filling phase of the micturition cycle (Wyndaele, 2001).

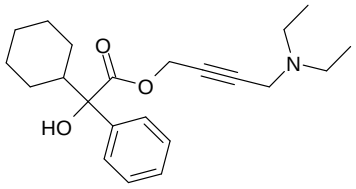
Anticholinergic drugs are the recommended first-line pharmacological therapy for overactive bladder (Tiwari and Naruganahalli, 2006) but their clinical use is compromised by their lack of selectivity, which leads to classical antimuscarinic side effects such as dry mouth, tachycardia and blurred vision. (Hegde et al., 2004). Muscarinic acetylcholine receptors are the predominant receptor system controlling bladder contractility (Andersson and Yoshida, 2003). Five subtypes of muscarinic receptors (M_1-M_5) have been cloned and pharmacologically characterized, which belong to the GPCR family. Among them, M_1, M_3 and M_5 receptors couple to G_q to mediate phosphoinositide hydrolysis, whereas the M_2 and M_4 receptors couple to $G_{i/o}$ to mediate adenylyl cyclase inhibition (Peralta et al., 1988). Moreover, studies performed in human urinary bladder have shown that carbachol-induced contraction via M_3 receptors, largely depends on Ca^{2+} entry through nifedipine-sensitive channels and activation of rho-kinase (Schneider et al., 2004), in addition to the IP_3 pathway.

Competition binding (Goepel et al., 1998) and immunoprecipitation studies with subtype-selective antisera (Wang et al., 1995) have demonstrated that although M_2 receptors account for 75-80% of the bladder's muscarinic receptor population, M_3 subtype which comprises only 20-25%, seems to be the main responsible for detrusor (urinary bladder smooth muscle) contraction. Other studies corroborate this conclusion (Chess-Williams et al., 2001; Fetscher et al., 2002). Further data supporting the functional role of M_3 receptors come from studies using M_3 knockout mice (Matsui et al., 2000), which demonstrated that M_3 receptors were the predominant muscarinic receptor subtype mediating bladder contractions. The residual direct contractile responses that persisted in M_3 knockout mice were completely lost in M_2/M_3 double knockout mice, suggesting that M_2 receptors play a minor role in carbachol-induced urinary bladder contractility (Stengel et al., 2000; Matsui et al., 2002). It has been suggested that M_2 receptors can also influence bladder contraction by enhancing the contractile response to M_3 receptor activation, through an interactive response of those receptors, (Ehlert et al. 2005). However, blockade of M_3 receptor seems to be sufficient to show efficacy on abnormal detrusor overactivity (Stevens, 2007).

The mammalian heart expresses mainly M_2 muscarinic receptors (Caulfield, 1993). Stimulation of muscarinic receptors within the heart, specifically the M_2 subtype, modulates pacemaker activity and AV conduction and directly (atrium) or indirectly (in ventricle) the force of contraction (Andersson et al, 2007). The M_2 receptor subtype has been reported to be the main responsible for muscarinic receptor-dependent bradycardia, whilst functional M_3 receptors are not involved in muscarinic receptor-dependent atrial rate reduction (Stengel et al., 2000; Stengel et al., 2002). Likewise, Fisher et al. (2004) have recently reported that bradycardia induced by vagal stimulation or methacholine administration was utterly abolished in M_2 receptor knockout mice, but

remained unaltered in M_3 receptor deficient mice. All other receptor subtypes are also expressed in cardiac tissues from different species, such as humans, dogs, guinea pigs and rats, but their role, if any, in the heart is not completely understood yet.

The reported critical role of M_3 receptors in mediating human detrusor contractility has led to the search for muscarinic receptor antagonist with greater selectivity for the M_3 receptor. We have developed a novel substituted quinuclidine derivative, SVT-40776 (Farrerons et al., 2002; Salcedo et al., 2002), highly selective for human M_3 over M_2 receptors. SVT-40776 has successfully finished Phase I clinical trials and is currently undergoing Phase II clinical studies for the treatment of overactive bladder. The objective of the present study was to characterize the binding properties of SVT-40776 and compare them with those of currently available antimuscarinic agents such as oxybutynin, tolterodine, darifenacin and solifenacin (see structures below). To that aim, SVT-40776 receptor affinity and selectivity was determined by [^{3}H]NMS binding competition and direct binding of [^{3}H]SVT-40776 to CHO cells stably expressing human muscarinic receptors. In addition, we studied SVT-40776 binding behaviour and the kinetics of its interaction to human M_3 and M_2 receptors.



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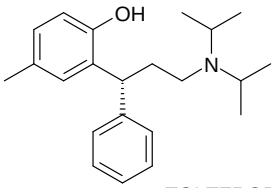

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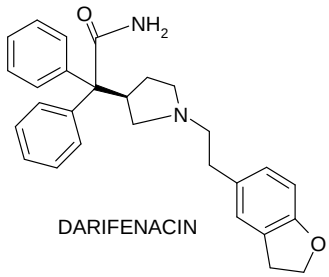

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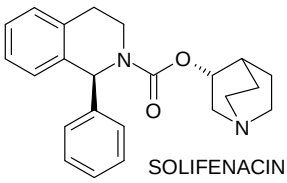

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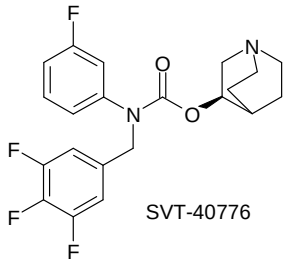

SVT-40776

SVT-40776 exhibited a high affinity for the human M_3 receptor, where it behaved as a competitive reversible antagonist. The compound showed a higher degree of selectivity for M_3 over M_2 receptor subtype, when compared to the marketed antimuscarinic drugs. The binding properties of SVT-40776 suggest that this novel compound could render improved efficacy and a safer cardiovascular profile over currently available treatments for overactive bladder.

Methods

Saturation binding studies with [^{3}H]NMS

Saturation curves were performed incubating different concentrations of [^{3}H]NMS with 25 µg of the membrane preparation and increasing concentrations of antagonist in PBS. Incubation was carried out in a total volume of 200 µL at 25ºC for 1 h. Membrane homogenates were preincubated for 1.5 h with the antagonist before adding the radioligand. Non-specific binding was defined in the presence of 5 µM atropine. The binding reaction was terminated by filtration over presoaked (0.5% PEI) 96-well glass filter plates (Millipore; type FC) using a Multiscreen vacuum manifold (Millipore). The filters were washed 3 times with ice-cold 50 mM Tris-HCl, 0.9% NaCl (pH 7.4), dried and 10 µL of Betaplate scintillation liquid (PerkinElmer) were added to each well. The retained radioactivity was quantified in a liquid scintillation counter (Microbeta® TriLux; PerkinElmer).

Saturation binding studies with [^{3}H] SVT-40776

Different concentrations of [^{3}H]SVT-40776 were incubated with 20-25 µg of the membrane preparation in PBS. Incubation was carried out in a total volume of 200 µL for 1 h at 25ºC and the non-specific binding was defined in the presence of 5 µM atropine. As described for the saturation binding assays with [^{3}H]NMS, the binding reaction was terminated by filtration followed by washing with ice-cold 50 mM Tris-HCl, 0.9% NaCl, pH 7.4. Once the filters were dried, 10 µL of Betaplate scintillation liquid (PerkinElmer) were added in each well. Finally, the retained radioactivity was quantified in a liquid scintillation counter (Microbeta® TriLux; PerkinElmer).

Competition studies with [^{3}H]NMS

Competition curves were determined incubating 0.5 nM [^{3}H]NMS with 20-25 µg of the membrane preparation and different concentrations of antagonist in a total volume of 200 µL for 1 h at 25ºC. Non-specific binding was defined in the presence of 5 µM atropine. The reaction was stopped and radioactivity quantified as described above.

Competition kinetic studies with [^{3}H]NMS

CHO-M_3 and CHO-M_2 membrane preparations were allowed to equilibrate with SVT-40776 for 3 h at 25ºC in continuous shaking. Specific binding was determined with [^{3}H]NMS following initiation of dissociation with a 50-fold dilution. Non-specific binding was defined in the presence of 5 µM atropine. The reaction was terminated by filtration over presoaked printed-filtermat (Wallac) filters using a harvester (Brandel). Filters were sealed in plastic bags containing 3 mL of Betaplate scintillation liquid and the retained radioactivity was determined.

Association and dissociation kinetic experiments with [^{3}H]SVT-40776

For association assays, CHO-M_3 membranes were incubated with 1 nM [^{3}H]SVT-40776 for 1 h at 25ºC. All reactions were initiated by the addition of the radioligand. Free [^{3}H]SVT-40776 was separated at multiple time points to build association kinetic curves. For dissociation experiments, membrane homogenates were allowed to reach equilibrium with 1 nM [^{3}H]SVT-40776 (approximately 1 h at 25ºC). Dissociation was initiated by the addition of 1 µM of cold SVT-40776, and bound [^{3}H]SVT-40776 was measured at different time points. In both kinetic studies, non-specific binding was

defined in the presence 5 µM atropine. The reaction was terminated by filtration and radioactivity quantified (as previously described).

Measurement of intracellular Ca^{2+} mobilization

CHO-K1 cells stably transfected with cDNA encoding the human M_3 receptor subtype were obtained from the National Institute of Health. Cells were grown and maintained in Ham's F12 medium containing 1 mM glutamine (Sigma), 1% penicillin/streptomycin (Sigma), 5% calf serum (PAA) and 5% fetal bovine serum (Clinus), and were grown for 3-4 days at 37ºC in a humidified incubator containing 5% CO_2, 95% O_2. For calcium mobilization assays, the cell line was grown to 80% confluence and after washing, cells were harvested by incubation with 4.8 mM EDTA in HBSS supplemented with 0.1 mM HEPES and 17 mM NaCl (pH 7.4) for 15 min at 37ºC and centrifuged at 4ºC. CHO-M3 cells were washed with buffer A (148 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, 1 mM glucose) and 107 cells/mL were incubated in the same buffer containing 0.1% bovine serum albumin, 2.5 mM probenecid and 4 µM Fluo3-AM for 45 min at 37ºC in continuous shaking. After the incubation, the cells were washed and resuspended at "2x107" cells/mL in buffer A containing 2.5 mM probenecid. The cell suspension was then incubated for 15 min at 4ºC to allow complete de-esterification of intracellular AM esters. Different concentrations of antagonist, 10^6 cells and buffer A were added to each black microtiter well and incubated for 15 min. Next, ACh was injected at a final concentration of 0.3 µM. Intracellular Ca^{2+} mobilization was monitorized for 3 min by spectrofluorometry in a Victor^2 (PerkinElmer) with an excitation wavelength of 490 nm and an emission wavelength of 510 nm.

Receptor binding profile

The general receptor binding profile of SVT-40776 was evaluated at CEREP (Celle l'Evescault, Poitiers, France). The activity of the compound in displacing approximately 30 different native or human recombinant binding sites was studied at a concentration of 1 µM in duplicate using radioligand binding techniques. The most common subtypes of the following receptors were investigated: angiotensin, bradykinin, cholecystokinin, endothelin, melanocortin, neurokinin, adenosine, adrenergic, benzodiazepine, dopamine, GABA, glutamate, histamine, nicotinic, opiate, serotonergic, sigma, glucocorticoid, vanilloid and all amine transporters.

Data analysis

Data points derived from the specific binding were analyzed by non-linear curve-fitting using Prism 4.0 (GraphPad Software®, San Diego, CA, USA). Binding parameters were obtained as the best-fit values for the data using the least-squares method.

Intracellular calcium mobilization was defined as a peak increase over the prestimulation levels in response to ACh. Data points derived from the maximum values of the peaks were taken to calculate the antagonist inhibition curves using a non-linear curve fitting.

Dissociation rate for unlabeled SVT-40776 was obtained after transforming the data from the competition kinetic experiments and expressed as % inhibition of [^{3}H]NMS. The transformed data were fitted to a one exponential decay and the k_{off} calculated.

[^{3}H]SVT-40776 kinetic parameters were calculated using Prism 4.0. Dissociation data were fitted to a one phase exponential decay function to calculate the k_{off} rate. [^{3}H]SVT-40776 association data were fitted to a one site binding function to calculate the observed rate constant k_{obs}. The association constant, k_{on} was calculated using Equation

1: $K_{on}= (k_{obs}-k_{off})/[radioligand]$, where the k_{off} value used was previously determined from dissociation rate experiments.

Apparent affinities values of antagonists (K_b) were calculated using Equation 2, the functional equivalent of the Cheng-Prusoff equation (Craig, 1993; Cheng, 2001): $K_b= IC_{50}/[1+(A/EC_{50})]$, where A is the agonist concentration and EC_{50} is the EC_{50} derived from agonist concentration-response.

Materials

[^{3}H]NMS (79-84 Ci/mmol) and [^{3}H]SVT-40776 (15 Ci/mmol) were obtained from Amersham Biosciences (United Kingdom). 4-DAMP mustard was purchased from Research Biochemicals International (Natick, MA, USA). Atropine, acetylcholine (ACh) and oxybutynin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). SVT-40776, darifenacin, solifenacin and tolterodine were synthesized in Laboratorios SALVAT (Esplugues de Llobregat, Spain). Chinese hamster ovary (CHO-K1) cell membranes containing human muscarinic receptors (M_1-M_5) were obtained from PerkinElmer (MA, USA). Hanks' balanced salt solution (HBSS) was purchased from Sigma-Aldrich Inc. and phosphate buffered saline (PBS) was prepared in-house (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4).

Results

SVT-40776 receptor affinity and selectivity

The affinity and selectivity of SVT-40776 for the human M_3 mACh receptor subtype was first determined by [^{3}H]NMS receptor binding competition in CHO cell membranes containing muscarinic receptors. Receptor binding affinities were also determined for a range of well-known muscarinic antagonists such as oxybutynin, tolterodine, darifenacin and solifenacin. Previously, equilibrium dissociation constants (K_d) values for [^{3}H]NMS at M_1-M_5 were determined by saturation binding analysis, in order to calculate the affinities (K_i) of the competing compounds using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). The affinity and selectivity (expressed as K_i M_x/M_3 ratio) of the antagonists for the different mACh receptor subtypes are summarized in Table 1.

SVT-40776 exhibited high affinity in the sub-nanomolar range (K_i= 0.19 nM), for the human M_3 muscarinic receptor. Particularly, SVT-40776 displayed the highest selectivity (203-fold) for the M_3 vs. the M_2 receptor subtype, in comparison with the other antagonists tested. Darifenacin also showed marked M_3 vs. M_2 selectivity (29.4-fold), while oxybutynin (5.7-fold) and solifenacin (5.9-fold) showed less selectivity for the M_3 receptor, and tolterodine completely lacked of selectivity (see Table 1). [^{3}H]SVT-40776 binding experiments were performed to determine the equilibrium binding parameters K_d and B_{max} (Table 2). Its specific binding to M_1, M_3, M_4 and M_5 receptors was more than 90% of total binding. The representative saturation curves in human M_1, M_3, M_4 and M_5 receptors are shown in Figure 1. The non-linear adjust of the data fitted well with the presence of a single homogeneous population of sites in each cell line. It was not possible to determine K_d and B_{max} for [^{3}H]SVT-40776 binding to the M_2 receptor, as there was no specific binding of the radioligand, even at concentrations as high as 1 μM. The affinity (expressed in terms of K_d value) of [^{3}H]SVT-40776 for M_3 (K_d= 0.54 nM) correlated well with the K_i value (K_i= 0.19 nM) obtained in the [^{3}H]NMS binding experiments, indicating that the compound binds specifically and selectively to this receptor. The K_d obtained for M_1, M_4 and M_5 (in all cases, higher that 1 nM) were also in good agreement with the affinities found in the [^{3}H]NMS competition assays.

In order to seek for other potential unwanted activities, SVT-40776 was tested on a battery of peptide receptors (angiotensin, bradykinin, cholecystokinin, endothelin, melanocortin, neurokinin), and non peptide receptors (adenosine, adrenergic, benzodiazepine, dopamine, GABA, glutamate, histamine, nicotinic, opiate, serotonergic, sigma, glucocorticoid, vanilloid and all amine transporters), at a single high concentration (1 μM), which represents 2000-fold the affinity for the human M_3 muscarinic receptor. The binding of SVT-40776 was negligible in all cases (data not shown), hence supporting the selectivity of SVT-40776 for the M_3 receptor.

SVT-40776 effect on Ca^{2+} mobilization induced by ACh

To elucidate the functional behavior of SVT-40776 at the human M_3 receptor, the effect of the compound on Ca^{2+} mobilization induced through the activation by an agonist such as ACh was determined. Firstly, preliminary experiments were performed to find the proper concentration of ACh, capable of yielding a functional response and whose inhibition could be accurately quantified. The concentration of 0.3 μM ACh was chosen. Then, CHO cells expressing human M_3 receptors were incubated for 15 min with different concentrations of SVT-40776 or other antagonists such as darifenacin, solifenacin and tolterodine. Afterwards, 0.3 μM ACh were added to the mixture and Ca^{2+} mobilization was recorded for 3 min. Figure 2 shows a representative experiment of SVT-40776 muscarinic antagonist activity on Ca^{2+} mobilization. SVT-40776 inhibited ACh-induced Ca^{2+} mobilization through human M_3 receptors in a concentration-dependent fashion. Subsequently, functional affinity constants were calculated for each antagonist and compared to their respective binding affinity constants. As shown in Table 3, SVT-40776 was the most potent compound in inhibiting ACh-induced Ca^{2+} mobilization (K_B= 0.28 nM), when compared to the rest of muscarinic antagonists (7-fold, 4-fold and 7-fold more potent compared to darifenacin, solifenacin and tolterodine respectively). The differences found in the antagonists' capability in inhibiting ACh-induced Ca^{2+} mobilization through human M_3 receptors have a good correlation with its binding affinities for the M_3 receptor.

Characterization of SVT-40776 binding behaviour

To investigate the reversible nature of SVT-40776 binding compared with other antagonists, CHO-M_2 and CHO-M_3 cell membranes were preincubated with the compounds and further diluted in the presence of 0.5 nM [^{3}H]NMS. An antagonist concentration achieving more than 60% inhibition in the competition curves was chosen. As shown in Figure 3, [^{3}H]NMS binding was recovered after dilution in samples preincubated with SVT-40776, darifenacin or tolterodine, suggesting that these antagonists behaved as reversible ligands of both M_2 (Figure 3A) and M_3 (Figure 3B) receptors. As expected, the activity was not recovered after 4-DAMP mustard preincubation and dilution, clearly indicating an irreversible interaction with both receptors. Besides, the recovery of the [^{3}H]NMS binding after a 50-fold dilution was in good agreement with that found in the competition curves in all cases (data not shown).

To elucidate the type of interaction of SVT-40776 with the human M_3 and M_2 receptors, [^{3}H]NMS saturation curves were performed in the presence of different antagonist concentrations. The presence of SVT-40776, darifenacin, solifenacin and tolterodine produced concentration-dependent rightward shifts of [^{3}H]NMS saturation curves in M_3 receptor when compared to that obtained in the absence of antagonists (data not shown). These displacements were accompanied by a concomitant increase in the K_d value without significantly changing the B_{max} value in the case of SVT-40776 (Table 4), darifenacin, solifenacin and tolterodine for the M_3 receptor (Table 5), which was

compatible with a competitive binding. As expected, 4-DAMP mustard induced a concentration-dependent reduction in the B_{max} value without modifying the K_d parameter, thus confirming its irreversible behaviour. Additionally, [^{3}H]NMS saturation studies were carried out with CHO-M_2 cell membranes preparations to further examine SVT-40776 binding features. As shown in Table 4, SVT-40776 displaced [^{3}H]NMS from the binding site, increasing its K_d without any change in B_{max} value. This indicated that the compound behaved as a competitive antagonist of the M_2 ACh receptor too. However, this effect was only observed from a 10 nM concentration on, since at lower concentrations no changes in K_d and B_{max} parameters were detected.

Characterization of SVT-40776 binding kinetic parameters

In order to study the kinetics of SVT-40776 interaction to human M_3 and M_2 receptors, the radiolabeled compound was used. The interaction of [^{3}H]SVT-40776 to M_3 receptors was time-dependent and allowed to directly determine its association and dissociation, showing that the equilibrium was achieved at 10 min (Figure 4A). The kinetic parameters are summarized in Table 6. The kinetically derived K_d (K_d= 0.16 nM), calculated as the ratio K_{off}/K_{on} was in good agreement with the parameters determined from [^{3}H]NMS competition (K_i= 0.19 nM) and [^{3}H]SVT-40776 saturation (K_d= 0.54 nM) experiments.

As mentioned above, it was not possible to carry out the experiments with the M_2 receptor since no specific binding was detected with these membranes. The dissociation of unlabeled SVT-40776 from M_2 receptors was calculated indirectly by measuring the rate of [^{3}H]NMS association after dilution to induce antagonist displacement. The results were represented as the inhibition of [^{3}H]NMS association (Figure 5), which reflects the percentage of receptors occupied by SVT-40776. Simultaneously, the apparent K_{off} of SVT-40776 from human M_3 receptors was also determined (Table 6). The resulting dissociation rate constants (K_{off}) of SVT-40776 from human M_2 and M_3 receptors were 1.15 and 0.07 min^{-1}, respectively; indicating that the compound dissociates 16-fold faster from M_2 than from M_3 (Figure 5). In addition, there was an excellent correlation between the [^{3}H]SVT-40776 dissociation rate measured directly (K_{off}= 0.06 min^{-1}), and the one determined from the kinetic assay with unlabeled SVT-40776 (K_{off}= 0.07 min^{-1}).

Discussion and Conclusions

Muscarinic antagonists are the mainstay in pharmacological therapy for the management of overactive bladder. They act by blocking the parasympathetic acetylcholine pathway and thus reducing the intensity of detrusor muscle contraction. However, their lack of selectivity has lead to classical antimuscarinic side effects such as dry mouth, tachycardia and blurred vision, limiting their efficacy and tolerability.

Efficacy of antimuscarinic drugs for the treatment of OAB has been evaluated in several clinical trials. A systematic review of 32 randomized controlled trials conducted by Herbison et al (2003) concluded that antimuscarinic agents produced significant improvements in OAB symptoms compared with placebo, even though the clinical relevance of these differences was uncertain. A recent update of a Cochrane systematic review has corroborated the efficacy of anticholinergic medication, also suggesting that improvements in symptoms may be associated with modest improvement in quality of life (Nabi et al., 2006). The overall concept of improvement in quality of life was introduced to support a reported efficacy that did not reflect the real limited effectiveness, a handicap that none of lately launched treatments has been able to overcome. From a rational point of view, logical questions could arise: Is efficacy

compromised because the dose level is limited? Is the dose limited because of the probability of adverse event occurrence? The incidence of typical muscarinic adverse events such as constipation or dry mouth has been shown to increase with dose (Chapple et al., 2005; Hay-Smith et al., 2005). However, cardiac effects due to M_2 blocking, which would be unacceptable for a non-life-threatening condition, have been clearly underreported.

The potential benefits of an M_3 selective drug, in terms of tolerability and safety, have been suggested (Andersson, 2002), which should avoid M_2 related cardiac effects. The cardiovascular safety of the marketed muscarinic antagonists darifenacin and tolterodine was evaluated as part of a double-blind trial that assessed efficacy of therapy (Olshansky et al., 2006; Romanzi et al., 2005). Tolterodine significantly increased heart rate in comparison with darifenacin and placebo. The percent of patients with an increase in heart rate of $\geq$ 5 bpm from baseline to last observation was significantly greater with tolterodine (39.3%) than placebo (23.2%, $p<0.01$). Tiotropium, a well known non-selective muscarinic antagonist developed for the treatment of chronic obstructive pulmonary disease (COPD), showed in a pooled clinical trial analysis, a slightly elevated risk of tachycardia when compared to placebo (Kesten et al., 2005). Moreover, Barr et al. (Barr et al., 2006) published a meta-analysis of available randomized trials, in which, among the adverse events reported, the authors pointed out that the frequency of arrhythmias was significantly higher with tiotropium than with placebo.

In the present study we have reported the binding characterization of a novel quinuclidine derivative with antimuscarinic properties, SVT-40776, (Farrerons et al., 2002; Salcedo et al., 2002) developed for the treatment of overactive bladder. We have characterized the binding features of this compound to human muscarinic receptors as well as its functional behaviour in CHO cells stably transfected with the receptors. SVT-40776 binding properties have been compared with those of the marketed antimuscarinic agents oxybutynin, tolterodine, darifenacin and solifenacin

In competition binding studies with [^{3}H]NMS in CHO cell membranes expressing human muscarinic receptors, SVT-40776 showed the highest affinity (K_i= 0.19 nM) and selectivity (203-fold vs. M_2) for the human M_3 receptor subtype, in comparison with other muscarinic antagonists, such as oxybutynin, tolterodine, darifenacin and solifenacin.

In addition, SVT-40776 was tested on a battery of peptide receptors (angiotensin, bradykinin, cholecystokinin, endothelin, melanocortin, neurokinin), and non peptide receptors (adenosine, adrenergic, benzodiazepine, dopamine, GABA, glutamate, histamine, nicotinic, opiate, serotonergic, sigma, glucocorticoid, vanilloid and all amine transporters), at a concentration 2000-fold higher than its affinity for M_3. The binding of SVT-40776 to these receptors was negligible, supporting the lack of other non-muscarinic activities.

In vitro functional studies have shown that SVT-40776 totally inhibits ACh-induced Ca^{2+} mobilization through human M_3 receptors, conferring SVT-40776 full antagonist behavior. SVT-40776 was more potent in blocking ACh-induced Ca^{2+} mobilization (Kb= 0.28 nM), in comparison to the muscarinic antagonists tested, which is in good correlation with their binding affinities for the M_3 receptor. The K_i value for an antagonist should theoretically be equivalent to the Kb determined in an *in vitro* functional experiment when similar assay conditions are used. In our case, the small differences found between K_i and Kb values, respond to different assay conditions as well as different incubation buffers used in those experiments.

The kinetics of SVT-40776 binding to human M_2 and M_3 receptors was studied by analyzing the association and dissociation rates using the radiolabeled compound. The obtained results indicated that the association of SVT-40776 to human M_3 receptor was faster than its dissociation. No specific binding to the M_2 receptor was detected using [^{3}H] SVT-40776, and consequently, the dissociation of unlabeled SVT-40776 from M_2 receptors had to be determined indirectly using [^{3}H]NMS. The calculated dissociation rate constants of SVT-40776 from human M_2 and M_3 receptors indicated that SVT-40776 dissociates 16-fold faster from M_2 than from M_3 receptors. These data suggest that the *in vivo* functional M_3 vs. M_2 selectivity of SVT-40776 can be even higher than that described in binding studies. As SVT-40776 lacks affinity for M_2 receptors and has fast dissociation from this receptor, it is therefore not expected to cause any harmful M_2-related cardiac effect as a prominent role of M_2 receptor has been demonstrated in heart rate increase.

There is an excellent correlation between M_3 dissociation rates (K_{off}) determined directly with [^{3}H] SVT-40776 (0.06 min^{-1}) and indirectly with unlabeled SVT-40776 (0.07 min^{-1}), reinforcing the consistency of both results. In addition, the kinetically derived K_d, calculated as the ratio K_{off}/K_{on} (K_d= 0.16 nM) was in good agreement with the values obtained from [^{3}H]NMS competition (K_i= 0.19 nM) and [^{3}H]SVT-40776 saturation (K_d= 0.54 nM) experiments, confirming the accuracy of the obtained results and providing a good validation of the methods (Dowling and Charlton, 2006).

Muscarinic antagonists differ in their binding affinity for the five human recombinant muscarinic receptors (Table 1). While tolterodine does not discriminate between the five subtypes, oxybutynin shows marginal selectivity for M_3 over M_2/M_5 subtypes and do not discriminate between M_3 and M_1/M_4 subtypes; solifenacin shows marginal selectivity between M_3 and M_2 subtype and do not discriminate between M_3 and $M_1/M_4/M_5$ subtypes; darifenacin has a high degree of selectivity for M_3 over M_1/M_2 subtypes and modest selectivity for M_3 over M_4/M_5 subtypes. Differently, SVT-40776 possesses high degree of selectivity for M_3 over M_2 subtype and marginal selectivity for M_3 over $M_1/M_4/M_5$ subtypes (Table 1).

Due to its high M_3 affinity, it could be expected that SVT-40776 could block M_3 receptors in glands and other (non-bladder) smooth muscles rich in this receptor subtype. The salivary glands are known to contain M_1 and M_3 receptors, whose activation is crucial for salivary secretion (Culp et al. 1996). Studies realized by Gautam et al. using M_1/M_3 receptor double-KO mice, showed that pilocarpine-induced salivary secretion was abolished in those mice (Gautam et al. 2004), which is consistent with the concept that muscarinic receptor mediated stimulation of salivary secretion is mediated by a mixture of M_1 and M_3 receptors. In order to assess a possible dry mouth side effect produced by SVT-40776, we performed studies in our lab comparing inositol phosphate accumulation in response to muscarinic receptor activation in mice and human bladder and salivary glands. SVT-40776 exhibited a high selectivity for bladder over salivary gland (23-fold in mice tissues and 14-fold in human samples) (Balsa et al. 2005, Enrich et al. 2008, paper in preparation).

In addition to present a good uroselectivity, an antimuscarinic drug developed to treat OAB and which will be addressed mainly to aged patients, should avoid CNS effects. Although all five muscarinic receptor subtypes are expressed in the brain, M_1 receptors play a major role in higher cognitive processes such as learning and memory (Caccamo et al. 2006). Thus, antagonism of M_1 receptor by antimuscarinics could produce important undesirable CNS side effects. Taking into account the moderate affinity showed by SVT-40776 on M_1 receptor subtype, we investigated the drug penetration into the brain, in dog and mouse after oral administration of [^{14}C]SVT-

40776 (data not shown). The obtained results showed very low levels of radioactivity in dog and mouse brain (17-fold lower than in urinary bladder) suggesting a low penetration of SVT-40776 through the blood-brain.barrier (Lagunas et al. 2005).

Although it is accepted the role of M_2 and M_3 receptors located in the detrusor muscle on bladder contraction, recent studies have demonstrated their presence on the human bladder mucosa/urothelium (Mansfield et al. 2007). Molecular reverse transcription (RT)-PCR studies have shown the expression of mRNA for M_2 and M_3 receptors in detrusor and mucosa from control patients as well as in detrusor and mucosa of patients with refractory idiopathic detrusor overactivity. The studies showed a significant less expression of M_3 receptor mRNA in the mucosa but not in the detrusor of overactive bladder (Mansfield et al. 2007). Although the precise role of muscarinic receptors in the bladder mucosa is not clear yet, it has been suggested that their stimulation could release an unknown urothelial-derived inhibitory factor (UDIF) able to modulate the detrusor contractile activity (Hawthorn et al. 2000, Scott et al. 2005, Mansfield et al. 2007, Murakami S et al. 2007). Urothelial muscarinic M_2 receptors could mediate UDIF release (Scott et al. 2005). It can be thought that this inhibitory factor could be decreased in the overactive bladder, causing at least in part, an increase in bladder contraction. In that case, an antagonistic activity on M_2 receptor would decrease efficacy for an antimuscarinic agent in the treatment of overactive bladder. On the contrary, SVT-40776, lacking activity on M_2 receptor, would have an advantage in the treatment of the syndrome. Further studies are needed to establish the specific bladder areas affected by antimuscarinics' therapeutic effects.

Clinical information reported thus far comes from five placebo-controlled Phase I studies involving a total of 96 healthy volunteers (young males and post-menopausal females), 80 of which received SVT-40776. No serious adverse events or clinically relevant changes in vital signs, ECG or laboratory tests were reported. The incidence of adverse events when subjects received 0.125 mg of SVT-40776, the nearest to the expected range of therapeutic doses, was similar to placebo, suggesting a good safety and tolerability profile (Viayna et al., 2004; Rigau et al., 2005). Preclinical and clinical data suggest that the expected therapeutic dose of SVT-40776 could be around 0.1 mg/day. These doses are between 40 and 100-fold lower than the therapeutic doses of tolterodine, oxybutynin, solifenacin and darifenacin (Hegde, 2006).

The present study robustly shows that SVT-40776 is the most potent and selective M_3 vs. M_2 receptor antagonist described for OAB treatment. Furthermore, SVT-40776 behaves as a reversible competitive antagonist, which predicts a good tolerability profile regarding to antimuscarinic adverse effects. The compound has successfully completed Phase I clinical trials and it is currently undergoing Phase II clinical trials for the treatment of overactive bladder. Preliminarily encouraging clinical results in patients are being obtained. In conclusion, the *in vitro* binding characterization together with the available clinical data, strongly suggest that SVT-40776 could yield increased efficacy for the treatment of overactive bladder, expecting a better tolerability over existing treatments.

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Table 1. Affinity estimates of antagonists in [^{3}H]NMS radioligand binding assays at M_1-M_5 human mACh receptor subtypes.

Compound	Binding affinities (K_i values, nM)					Ratio			
	M_1	M_2	M_3	M_4	M_5	M_1/M_3	M_2/M_3	M_4/M_3	M_5/M_3
Oxybutynin	3.80 ± 0.40	9.15± 0.95	1.60 ± 0.30	1.68 ± 1.24	5.29± 0.65	2.4	5.7	1.1	3.3
Tolterodine	2.56 ± 0.53	2.21 ± 0.45	4.13 ± 1.75	1,77 ± 0.15	2.34 ± 1.10	0.6	0.5	0.4	0.6
Darifenacin	50.45 ± 3.95	88.65 ± 3.25	3.05 ± 0.25	20.90 ± 4.89	7.40 ± 2.40	16.6	29.4	6.9	2.4
Solifenacin	2.47 ± 0.50	43.23 ± 9.80	7.30 ± 1.40	2.40 ± 0.81	4.80 ± 0.72	0.3	5.9	0.3	0.7
SVT-40776	0.36 ±0.10	38.47± 16.40	0.19 ±0.09	0.31 ± 0.06	0. 38 ± 0.17	2.1	203	1.6	2.1

Values are expressed as the mean ± S.E.M. of, at least, two independent experiments, seven to ten points per curve, each point performed in duplicate. Hill slopes of competition curves were not significantly different from unity.

Table 2. [^{3}H]SVT-40776 binding at human mACh receptors. Recombinant human receptors were expressed in CHO-K1 cells.

Parameters	M_1	M_2	M_3	M_4	M_5
K_d (nM)	1.20 ±0.14	nSBD	0.54±0.09	1.03±0.10	1.56±0.01
B_{max} (fmols/mg)	5803±72	nSBD	1909±151	4822±274	9701±401
NSB (%)	6	---	6	5	4

Values are expressed as the mean ± S.E.M. of, at least, two independent experiments, seven to ten points per curve, each point performed in duplicate. Hill slopes of competition curves were not significantly different from unity. nSBD: no specific binding . NSB: % of non-specific binding.

Table 3. Comparison of [^{3}H]NMS binding affinities at hM_3 receptors and inhibition of hM_3 receptor-mediated elevation of intracellular Ca^{2+} produced by different antagonists.

Compound	**Binding assay (Ki; nM)**	**Ca^{2+} mobilization (Kb; nM)**
Darifenacin	3.05 ± 0.25	1.83 ± 0.43
Solifenacin	7.30 ± 1.40	1.09 ± 0.03
Tolterodine	4.13 ± 1.75	1.94 ± 1.11
SVT-40776	0.19 ± 0.09	0.28 ± 0.18

Intracellular Ca^{2+} mobilization was induced by 0.3 µM ACh. Antagonist binding affinities (Ki) to the M_3 receptor are included for comparison purposes. Values are expressed as the mean ± S.E.M. of, at least, two independent experiments, each point performed in duplicate.

Table 4. Effects of SVT-40776 on [^{3}H]NMS binding parameters at hM_2 and hM_3 receptors.

SVT-40776 (nM)	**hM_3**		**hM_2**	
	K_d	**B_{max}**	**K_d**	**B_{max}**
0	0.69±0.05	1364±32	1.03±0.1	4257±837
0.1	0.78±0.02	1291±103		
0.3	0.91±0.07	1251±118		
1	1.7±0.05**	1447±159		
3	3.9±0.5*	1458±43		
10	9.5±0.2**	1420±202	1.2±0.06	3930±83
30	27.5±14**	1493±720	1.5±0.3	4230±363
100			3.6±0.2**	4393±480
300			7.9±0.3*	4446±1629
1000			24±0.8**	4369±827
3000			63±0.7***	5208±607

K_d is expressed in nM and B_{max} in fmol/mg. Data are expressed as the mean ± S.E.M. of 2 independent experiments. Student's t test: *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. control curves in the absence of antagonist.

Table 5. Effects of different mACh receptor antagonists on [^{3}H]NMS binding parameters at the hM_3 receptor.

Concentration (nM)	Darifenacin		Solifenacin		Tolterodine		4-DAMP Mustard	
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
0	0.71±0.1	1463±111	1.1±0.7	1571±239	1.99±1.4	1639±253	1.4±0.5	1527±293
0.1	-	-	0.6±0.02	1321±93	-	-	1.5±0.4	1365±129
0.3	-	-	0.5±0.02	1293±95	-	-	1.3±0.1	1212±17
1	2.1±1.4	1526±24.4	0.7±0.09	1328±88	0.6±0.08	1247±302	1.3±0.4	773±203
3	1.2±0.5	1411±123	0.8±0.1	1278±110	-	-	1.0 ± 0.5	330±141*
10	1.5±0.3	1263±280	1.1±0.3	1240±101	0.9±0.15	1491±302	-	-
30	2.9±0.05**	1128±328	-	-	3.3±1.7	1525±408	-	-
60	7.7±1.1*	1171±396	-	-	-	-	-	-
100	11.9±4.0*	1488±615	8.4±3.7**	1499±358	8.4±3.2	1358±309	-	-
200	-	-	-	-	12±4.2*	1328±147	-	-
300	-	-	-	-	27±3.0**	1530±414	-	-
600	-	-	-	-	52±1.4**	1278±351	-	-

K_d is expressed in nM and B_{max} in fmol/mg. Values are expressed as the mean ± S.E.M. of 2-4 independent experiments. Student's t test: *$p<0.05$ and **$p<0.01$ vs control curves in the absence of antagonist.

Table 6. Kinetic parameters of SVT-40776 binding to hM_2 and hM_3 receptors.

	[^{3}H]SVT-40776		[^{3}H]NMS	
Parameter	**M_3 association**	**M_3 dissociation**	**M_3 dissociation**	**M_2 dissociation**
K_{obs} (min^{-1})	0.43 ± 0.05			
K_{on} (min^{-1} M^{-1})	$3.7x10^8$			
K_{off} (min^{-1})		0.06 ± 0.02	0.07 ± 0.01	1.15 ± 0.07
$t_{1/2}$ (min)	1.7 ± 0.2	13.6 ± 4.0		

Results were obtained either from [^{3}H]SVT-40776 or [^{3}H]NMS binding and are expressed as the mean ± S.E.M. of two independent experiments, each one performed in duplicate. Koff: dissociation rate constant; Kobs: observed rate constant; Kon= (Kobs-Koff)/[radioligand].

Figure legends

Fig. 1. Representative [^{3}H]SVT-40776 saturation curves to human M_1-M_5 receptors. Saturation curves of [^{3}H]SVT-40776 total (squares), non-specific (triangles) and specific (circles) binding to human (A) M_1, (B) M_3, (C) M_4 and (D) M_5 receptors. Each point was performed in duplicate.

Fig. 2. Effect of SVT-40776 on Ca^{2+} mobilization induced by acetylcholine in CHO-hM_3 cells. Typical recordings of the time-course of ACh (0.3 µM)-induced Ca^{2+} mobilization in the presence of increasing antagonist (SVT-40776) concentrations can bee seen, which values are indicated in the legend in µM. The arrow indicates the point of agonist injection in all cases.
Bottom: concentration-response relationship for the inhibition of ACh-induced intracellular Ca^{2+} mobilization by SVT-40776. Data represents the mean ± S.E.M of two independent experiments.

Fig. 3. Reversibility of the interaction of mACh antagonists with hM_2 and hM_3 receptors. Human M_2 (A) and M_3 (B) receptors were preincubated for 3 h with the antagonists at the concentrations indicated. Then, ligand displacement was achieved after a 50-fold dilution (open bars). The free binding sites were determined by [^{3}H]NMS (0.5 nM) binding and compared with those without dilution (closed bars). Results are expressed as the mean ± S.E.M. of 2-3 independent experiments.

Fig. 4. Kinetics of [^{3}H]SVT-40776 interaction to hM_3 receptor. (A) For ligand association, M_3 membranes were incubated with 1 nM [^{3}H]SVT-40776 for different time periods. (B) For dissociation experiments, membranes were preincubated with 1 nM [^{3}H]SVT-40776 for 1 h at 25ºC. Dissociation was initiated by the addition of 1µM of cold SVT-40776, and bound [^{3}H]SVT-40776 was measured at different time points. Data from one representative experiment are shown.

Fig. 5. Dissociation of unlabeled SVT-40776 from human M_2 and M_3 receptors. Dissociation of SVT-40776 from hM_2 and hM_3 receptors was determined by [^{3}H]NMS binding. CHO membranes expressing hM_2 (open circles) or hM_3 (closed circles) receptors were preincubated with SVT-40776 (at 300 nM for M_2 and 10 nM for M_3 receptor) for 3 h at 25ºC. After a 50-fold dilution, [^{3}H]NMS (0.5 nM) association was determined. Data from one representative experiment are shown.