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In vitro effects of the β_3-adrenergic agonist Mirabegron on the contraction of human prostate and bladder smooth muscle

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Content

List of abbreviations

5-ARIs	5α-reductase inhibitors
ANOVA	Analysis of variance
BOO	Bladder outlet obstruction
BPH	Benign prostatic hyperplasia
BPIPP	5-(3-Bromophenyl)-5,11-dihydro-1,3-dimethyl-1H-indeno[2',1':5,6]pyrido[2,3-d]pyrimidine-2,4,6(3H)-trione
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
DAG	1,2-diacylglycerol
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DO	Detrusor overactivity
EC_{50}	Half maximal effective concentration
EFS	Electric field stimulation
E_{max}	Maximum Effect (Here: contraction)
IC_{50}	Half maximal inhibitory concentration
IP3	inositol 1,4,5-trisphosphate
L-NAME	Nω-nitro-L-arginine methyl ester
LUTS	Lower urinary tract symptoms
OAB	Overactive bladder
PBS	Phosphate-buffered saline
PSA	Prostate-specific antigen
PVR	Post-void residual
Q_{max}	Maximum flow rate
TTX	Tetrodotoxin
TXA_2	Thromboxane A_2
TURP	Transurethral resection of prostate
UR	Urinary retention

List of Publications

Publications included in this thesis:

Huang R, Liu Y, Ciotkowska A, Tamalunas A, Waidelich R, Strittmatter S, Stief CG, Hennenberg M. Concentration-dependent alpha1-adrenoceptor antagonism and inhibition of neurogenic smooth muscle contraction by mirabegron in the human prostate. Front Pharmacol 2021;12:666047

Huang R, Tamalunas A, Waidelich R, Strittmatter F, Stief CG, Hennenberg M. Inhibition of full smooth muscle contraction in isolated human detrusor tissues by mirabegron is limited to off-target inhibition of neurogenic contractions. J Pharmacol Exp Ther 2022, in press

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Yu Q, Wu C, Chen Y, Li B, Wang R, **Huang R**, Li X, Gu D, Wang X, Duan X, Li S, Liu Y, Wu W, Hennenberg M, Zeng G. Inhibition of LIM kinase reduces contraction and proliferation in bladder smooth muscle. Acta Pharmaceutica Sinica B 2020, in press.

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Li B, Li P, Xia W, Yu B, Yu Q, Zhang B, **Huang R**, Wang R, Liu Y, Chen Z, Gan Y, He Y, Hennenberg M, Stief CG, Chen X. Phosphoproteomics identifies potential downstream targets of the integrin α2β1 inhibitor BTT-3033 in prostate stromal cells. Annals Translational Medicine 2021;9(17):1380.

Own contributions to publications

The collection of tissues and all organ bath experiments included in both publications were performed by the first author of both publications, Ru Huang. Cell culture experiments, which are included as a minor part in the first presented publication, were performed by co-authors and have a rather completive character, as they were performed to address reviewer comments for revision of the paper. Thus, the principal parts are the organ bath experiments, which provide the basis for all conclusions. The co-authors signed the "Cumulative Thesis" form and agreed to MD student Ru Huang to use the following publications to obtain the doctoral degree in Human Medicine. All authors confirm that the publications will not be used as part of another dissertation

1 Introduction

1.1 Preface

Lower urinary tract symptoms (LUTS) are a collective term for a range of symptoms including storage symptoms, voiding symptoms and post-voiding symptoms. They result in a considerable economic burden on healthcare systems, both directly and indirectly and in addition to reduced quality of life and complications [1, 2]. Storage symptoms, usually due to increased micturition stimuli or increased sensitivity to normal stimuli, include increased frequency of urination, nocturia, urinary urgency, and even urinary incontinence [3]. Voiding symptoms, which are mostly or nearly always caused by a urethral obstruction, including dysuria, hesitant urination, a weak or irregular urinary stream, urinary stream branching, terminal dribbling, and urinary retention [3]. Post-voiding symptoms include incomplete emptying, and post-void dribbling are also usually caused by urethral obstruction [4]. With these regularly occurring symptoms, LUTS always have a negative impact on the life quality of individuals [5, 6]. Both female and male patients can suffer from LUTS, but differences between genders exist, as LUTS are attributed to the urinary bladder in women, but can be caused by the bladder and prostate in men.

The pathophysiology of LUTS is not yet entirely clear [7, 8]. When medical scientists started to be aware of LUTS in the early stages of clinical medicine, BPH was considered as the exclusive cause of LUTS in men, while bladder symptoms were considered as those reserved to women [9, 10]. However, the modern and current understanding of LUTS encompasses a broader group of symptoms than BPH. As awareness of LUTS increased, it has been acknowledged that excessive activity of the bladder detrusor (overactive bladder, OAB) is another common reason for LUTS in addition to symptoms caused by obstruction of the urethra by BPH [11].

1.2 Epidemiology

As improved health care and increased life expectancy drive population ageing, the incidence of BPH and the prevalence of LUTS have increased rapidly. A survey among 30,000 individuals (USA 20,000; UK 7,500; Sweden 2,500) showed that the prevalence of LUTS ranges from 47.9% to 72.3% in men and from 52.5% to 76.3% in women [12]. Many studies have confirmed that the occurrence of LUTS is positively correlated with age [13]. Boyle et al. reported that almost 11% of married women and 10% of men complained of moderate to severe LUTS at the age of 40-49 years, while the percentage of LUTS increased by 30% in men at the age of 70-79 years [14]. Another recent study on males reported that 40% of them complained of moderate to severe LUTS when they were over 40 years old, and this proportion increased to 70% after the age of 70 [15]. Furthermore, in the group over 85, the percentage increased to 90% [14, 16, 17]. Due to the progressive character and the age-dependency of prevalence, together with the demographic transition in many countries, case numbers are increasing since years ago [11].

For BPH, an analysis based on data from the Integrated Healthcare Information System reported that among all men over the age of 50 in the United States, the incidence of BPH is 13.5%, ranking it the fourth among the top ten common diseases. When the statistical interval extended to men over 65, the incidence of BPH was 27.8%, which is still at the fourth of the most common disease [18, 19]. These numbers may reflect the lower range of estimations, considering that histological BPH may be found in 90% of men in the 9th life decade and that half of the patients with histological BPH may experience symptoms [13].

For OAB, an epidemiological study comprising five countries (Canada, Germany, Italy, Sweden, and the UK) based on the population over 40 years old suggested an overall OAB prevalence of 15.6% in males, 17.4% in females and an average prevalence of 16.6% [11]. Another survey conducted by the National Overactive Bladder Evaluation program in America included 5,204 participants over 18 years

who finished the computer-assisted telephone interview (CATI) questionnaire, from which 16.0% of males and 16.9% of females expressed that they have been annoyed by symptoms of OAB in the past four weeks before the telephone investigation. An average of 16.4% adults over 18 years old met the diagnostic criteria of OAB [20]. These two studies indicate a similar prevalence of OAB, around 16% to 17% in the adult population.

1.3 Aetiology

Many factors contribute to BPH and OAB, which are the two leading causes of LUTS. In LUTS suggestive of BPH, the hyperplastic prostate obstructs the urethral outlet, inducing a bladder outlet obstruction (BOO), which impairs urinary blow, bladder emptying, and triggers voiding symptoms [21]. OAB is another common cause of LUTS. By involuntary, spontaneous contractions in the bladder wall, the overactive bladder detrusor can cause symptoms like urgent urinary incontinence and other inconveniences during the storage phase [5, 22].

Many modifiable or non-modifiable factors can increase the risk of developing and progression of LUTS [21]. Age, metabolic diseases, and obesity are common risk factors, but also cystitis belongs to modifiable factors of LUTS [23-26]. At the same time, with new kinds of invasive surgical applications, some factors that were not available in the past can also lead to OAB. Pelvic organ prolapse (POP) is a common female problem, especially in those who have undergone spontaneous vaginal delivery. Most patients with advanced POP suffered from various LUTS. Approximately 200,000 women receive surgical repair each year in the United States of America [27, 28]. After surgical correction, previous LUTS may be corrected or may persist, and as another possibility, new symptoms can appear. In conclusion, POP itself has a high possibility to cause LUTS, and surgical treatment for POP can also result in LUTS [29].

Age is a crucial risk factor for BPH, as evidenced by numerous studies [13]. Exemplarily, a study addressing the progression of BPH with age followed up the development for a mean period of 5.9 years in 9,628 men [30]. This study followed patients with an International Prostate Symptom Score (IPSS) between 8 and 14 (mild LUTS) to understand the relationship between BPH progression and age. Specifically, the rate for progression from mild to moderate, and from mild to worse LUTS was examined for different age groups. The results showed that higher age groups had a higher rate of progression than the younger age groups [30]. Exemplarily, progression from mild to moderate or worse LUTS increased from 45.0 per 1,000 males per year in the age group of 40-54 year old men, to 72.2 per 1,000 in ≥75 years old men.

Metabolic syndrome and its single components are another important risk factor, and increasingly moving into the focus of clinical and preclinical research. An early study addressing metabolic factors and BPH included 158 BPH patients with or without metabolic syndrome [31]. Compared with the patients without metabolic syndrome, those with metabolic syndrome had significantly larger prostates (49.0 ml vs. 28.5 ml) and faster-growing prostates (1.019 ml/y vs. 0.699 ml/y). Specifically, patients with noninsulin-dependent diabetes mellitus, high blood pressure, obesity, low HDL cholesterol levels and fasting insulin levels had an annual prostatic growth rate of 49.2%, 16.6%, 36.0%, 31.0% and 27.6%, respectively [31].

Other reasons and risk factors include foreign bodies, infections, bladder tumours and intoxications, which can also cause LUTS. However, the leading reasons directly causing LUTS are BPH and OAB.

1.3.1 The Etiology of BPH

BPH, which causes BOO, is responsible for voiding symptoms such as dysuria,

hesitant urination, interrupted urinary stream, terminal dribbling, incomplete emptying, and eventually urinary retention. Overall, a decreased urinary flow rate, i. e. a weak urinary stream characterizes the voiding symptoms suggestive of BPH. BPH occurs when stromal and/or epithelial cells in the prostate proliferate, especially in the transition zone, with the processes thought to be influenced by sex hormones [32], resulting in prostate enlargement [21, 33]. Studies have shown that the balance between proliferation and apoptosis is significantly disturbed in BPH [32]. As a result, proliferative processes predominate, and the gland often becomes enlarged [8, 32, 34, 35].

In addition to prostate growth, increased prostatic smooth muscle tone is another mechanism contributing to urethral obstruction in BPH [36]. Prostate smooth muscle contraction is an essential target for medical treatment to relieve voiding symptoms [37]. These include α_1-adrenoceptor antagonists as the first-line option, which may improve symptoms by inhibiting adrenergic prostate smooth muscle contraction and subsequent smooth muscle relaxation in the prostate [36, 37]. Although increased prostatic smooth muscle tone can be triggered by activation of the adrenergic receptor on smooth muscle cells, there are also non-adrenergic receptors that can cause prostatic smooth muscle contraction. In recent years, increasing attention has been paid to non-adrenergic prostate smooth muscle contraction, and research addressing the associated inhibitory targets provides a basis for developing future drug treatments for LUTS [38].

It is generally believed that hormonally mediated cell proliferation and increased smooth muscle tension around the prostatic urethra can cause urethral compression that impairs bladder emptying and urine flow [13, 37]. When the dynamic resistance becomes unbalanced, this leads to obstruction-related voiding symptoms. However, recent studies have shown that inflammation and prostatic fibrosis also play a role in developing BPH associated with LUTS [39]. Inflammation of the prostate is related to

prostatic hyperplasia and prostatic fibrosis, which is considered as another cause of BOO, in addition to hormonal regulation [39]. Chronic inflammation can directly lead to prostatic tissue damage by activating cytokines and increasing the concentration of growth factors [39-42].

1.3.2 The Etiology of OAB

OAB is characterized by storage symptoms such as urinary urgency, often accompanied by frequency and nocturia, with or without urinary incontinence [43]. Regardless of the physical symptoms, the most relevant fact is that it affects the lives and social interactions of the sufferers, causing social disruption and triggering numerous negative emotions such as low self-esteem and anxiety. Other consequences include lower work productivity, higher unemployment and lower sexual satisfaction [5, 44, 45].

Involuntary detrusor contractions cause the symptoms of OAB during the urine storage phase [46]. These involuntary contractions are triggered by neurogenic, acetylcholine-induced stimulation of muscarinic receptors, called detrusor overactivity (DO) [46]. Causes of DO include myogenic factors and neurogenic factors. The myogenic theory assumes that the urge to urinate is triggered by spontaneous detrusor contractions, whereas the neurogenic theory assumes that the urgency for urinating is signalled by the central nervous system, which initiates detrusor contraction [7]. OAB and DO are not equivalent concepts, as the former is a diagnosis based on symptoms, whereas the latter is a description of urodynamics. However, there is a very close relationship between these two terms [47]. It has been concluded that about 64% of patients annoyed by OAB used to be confirmed to possess DO, and 83% of individuals with DO have symptoms suggestive of OAB [48].

With increased awareness, DO is no longer the only cause of OAB that has been

realized. In the early 2000s, evidence of abnormal signalling from urothelial/suburothelium cells emerged, supporting the bladder afferent hyperactivity hypothesis, which emphasizes that urothelial/suburothelium cells may trigger the urinary urgency as well [49]. Recently, the urethral hypothesis has also been proposed. The urethra is considered a possible source of OAB [50]. These different possible mechanisms provide new ideas to improve the diagnosis and treatment of OAB [5, 7, 44]. Altogether, OAB should be considered a complex, multifactorial symptom syndrome caused by various underlying pathophysiological mechanisms.

1.4 Pharmacological treatment of LUTS

1.4.1 The role of α_1-adrenoceptor antagonists in LUTS therapy

The α_1-adrenergic receptors belong to the family of G protein-coupled receptors and induce smooth muscle contraction after their activation [36, 51]. In vivo, they are involved in neurotransmission and regulate the sympathetic nervous system by binding the α_1-adrenergic agonists, which are assumed to be norepinephrine (the neurotransmitter) and epinephrine (the neurohormone) [52]. There are three subtypes (α_{1A}, α_{1B}, and α_{1D}) of α_1-adrenergic receptors that have been identified and characterized by differences in their function, radio ligand binding ability, structure and distribution [52-54]. Regarding the urinary tract, it is currently recognized that the α_{1A} subtype generally regulates smooth muscle tone in the prostate and bladder neck, whereas the α_{1D}-subtype may be involved in bladder function and in spinal cord innervations connected with the bladder [52, 55, 56]. Consequently, α_1-adrenoceptor antagonists (“α_1-blockers”) reduce prostate tone mainly by inhibiting the contractions of the prostate smooth muscle cells induced by the endogenously released noradrenaline.

Multiple clinical trials have shown that treatment with α_1-adrenoceptor antagonists can significantly ameliorate LUTS. It has been widely recognized that

α_1-adrenoceptor antagonists can reduce voiding symptoms by increasing the Q_{max} around 20-40% and reducing the International Prostate Symptom Score (IPSS) around 30-50% [37, 57-60]. While the full effects of α_1-adrenoceptor antagonists take weeks to develop, significant and clinically relevant effects occur already within hours to days [61-63].

However, even placebos may increase the Q_{max} at least by 15% and decrease the IPSS up to 30% and more [64-67]. Around 30-35% of patients respond to treatment with α_1-adrenoceptor antagonists with decreases of not more than 25%, resulting in overall disappointment about the treatment in 69% of patients [64, 68-70]. Consequently, only 35% of patients continue taking α_1-blockers 12 months after the first prescription [71, 72]. Finally, the low adherence to medical therapy represents a risk factor for hospitalization and surgery due to BPH [71]. Less surprisingly, α_1-adrenoceptor antagonists neither prevent acute urinary retention (AUR) nor the need for surgery due to BPH, since they did not reduce prostate size in long-term studies [37]. Moreover, they do not inhibit contractions of human prostate smooth muscle, if stimulated by non-adrenergic agonists [38]. Altogether, this demonstrates that the efficacy of the widespread applicated α_1-adrenoceptor antagonists still underlies high limits, so a more extensive work-up on new options with higher efficacy is mandatory [36, 38, 73, 74].

1.4.2 The role of muscarinic receptor antagonists in LUTS therapy

Muscarinic receptors comprise five subtypes, M_1-M_5, corresponding to different pharmacological actions [75]. As α_1-adrenoceptors, they also belong to the family of G protein-coupled receptors and elicit downstream effects by different G proteins. M_1, M_3, and M_5 receptors can trigger smooth muscle contractions by increasing intracellular calcium after mobilizing phosphoinositides that produce inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) via preferential coupling to $G\alpha_{q/11}$, whereas M_2 and M_4 receptors can reduce cyclic adenosine monophosphate

(cAMP) by coupling to $G\alpha_i$ inhibiting adenylyl cyclase activity [76].

OAB management usually begins with conservative therapies. The recommended therapy during the early stage of OAB consists of behavioural, dietary and lifestyle modifications, pelvic floor muscle training, and bladder retraining [45]. Muscarinic acetylcholine receptors are established drug targets for the treatment of storage symptoms in OAB [46, 77]. Antimuscarinics exert their effects by competitively antagonizing acetylcholine binding to M_2 and M_3 on the detrusor smooth muscle cells in the bladder wall. Thus, antimuscarinics are supposed to improve OAB symptoms by blocking detrusor contractions induced by acetylcholine, released from efferent nerve fibers and synthesized by the urothelium, affecting the muscarinic receptors in the urothelium and myofibroblasts [78-80].

For the treatment of OAB, antimuscarinics are the most commonly used and available drugs [79, 81]. They can significantly reduce urinary incontinence or the number of times to urinate. In a meta-analysis extracted from 83 trials, antimuscarinics were reported to reduce urinary incontinence by an average of 0.4 to 1.1 times per day and the micturition episodes by 0.5 to 1.3 times compared with placebo [82].

Although antimuscarinics effectively improve OAB symptoms, they have more side effects than other drugs used to treat LUTS, such as dry mouth, blurred vision, erythema, constipation, fatigue, increased sweating, urinary retention, and even central nervous system (CNS) adverse event [83, 84]. Thus, disproportional side effects along with limitations in efficacy contribute to discontinuation rates up to 90% within one year after the first prescription [85].

1.4.3 The role of 5α-reductase inhibitors (5-ARIs) in LUTS therapy

The 5α-reductase is an enzyme taking part in the metabolism of steroids in the

human body [86]. It is a crucial point for converting testosterone into dihydrotestosterone (DHT) [39]. DHT plays a vital role in prostate cell proliferation, inducing prostate enlargement regarded as a prime cause of BPH [87]. 5α-reductase inhibitors (5-ARIs) were introduced for the treatment of LUTS, based on the inhibition of androgen conversion, resulting in reduced DHT levels and thereby preventing the progression of prostate enlargement or even reducing the size of an enlarged prostate [86].

The current 5-ARIs have been widely used for clinical treatment of prostate enlargement, for nearly 30 years. It has been reported that the prostate size can be reduced by approximately 18-28% after six to twelve months of treatment with 5-ARIs alone [88]. For the short-term use (up to four weeks), the mean change in IPSS score was around -4, while the IPSS will change -5.0 and -5.5 after twelve and twenty-six weeks of use, respectively [89, 90]. The positive influence of 5-ARIs could be even higher after more than six months of treatment. After two to four years of use, patients with symptoms due to BPH experienced an improvement of IPSS around 15-30% and an increase in Q_{max} around 1.5-2.0 ml/s [91-93]. Because 5-ARIs can reduce the size of an enlarged prostate, it can decrease not only the risk of AUR but also the incidence of surgery [37].

Prostate-specific antigen (PSA) is produced by the epithelial cells of the prostate [94]. It is considered as a specific marker for prostate tissue and increases with BPH and with prostate cancer [94, 95]. A tiny amount of PSA can be released into the blood. Therefore, the presence of PSA can also be detected in human plasma [96]. DHT is the major promoter in the process of PSA synthesis [97, 98]. Therefore, taking 5-ARIs can lower the PSA concentration in the prostate, ultimately leading to a decrease in circulating PSA by approximately 50% [99]. Owing to the reduction of testosterone-dependent functions, 5-ARI has characteristic side effects in BPH patients, including decreased libido, gynecomastia, and erectile dysfunction [88, 93].

1.4.4 The role of β_3-adrenergic agonists in LUTS therapy

According to different sequences and structures, different distribution and expression patterns and finally due to divergent functions and pharmacological properties, three subtypes of β-adrenoceptors can be distinguished, including β_1, β_2 and β_3 [100]. β-adrenoceptors have been reported to exist in the human bladder, prostate and even urethra [56]. In the detrusor muscle and urothelium of the bladder, expression levels of the β_3-adrenoceptor may be far higher than expression levels of the other two subtypes [52, 101, 102]. β_3-adrenoceptor activation can activate adenylyl cyclase, increasing the intracellular cAMP concentration and thereby relaxing the smooth muscle cell [52]. Thus, β_3-adrenoceptor agonists were developed as one of the medical treatments by reducing the detrusor smooth muscle tone and promoting urine storage volume in OAB [103-106].

After β_3-adrenoceptor agonists were found to improve the storage symptoms of OAB, including frequency of micturition and urgency incontinence, the pharmacologic armamentarium has been expanded by approval of mirabegron [107-109]. Mirabegron is the first selective β_3-adrenoceptor agonist which was approved to be used in OAB patients by the American Food and Drug Administration [110]. Compared with placebo, it has been reported to cut down the mean incontinence episodes number per 24 h (MD -0.54), and it can also enhance the bladder storage capacity due to a reduction of the contractile ability of the bladder [107]. β_3-adrenoceptor agonists showed no remarkable adverse effects on voiding urodynamic parameters such as Q_{max}, bladder contractility index and detrusor pressure of the maximum flow. Moreover, the use of mirabegron is associated with better continuation rates compared to antimuscarinics [111]. However, although the efficiency of mirabegron has been proved in some clinic research, the underlying mechanism is still unclear, even though broad initial consent assumed it improves storage symptoms by bladder smooth muscle relaxation through activation of

β_3-adrenoceptors on detrusor muscle cells [112-114].

1.4.5 The role of phosphodiesterase (PDE) inhibitors in LUTS therapy

Phosphodiesterases (PDEs) are a family of enzymes that can degrade the second messenger molecules cAMP and cGMP, which both mediate smooth muscle relaxation. Acknowledging the amino acid sequences, substrate specificities, regulatory properties, pharmacological properties, and tissue distribution, the superfamily of PDE enzymes are classified into 11 families, namely PDE1-PDE11 [115]. PDE5 has been demonstrated to be expressed in the bladder detrusor, prostate tissue, and ureter in the urinary tract [116]. PDE5 inhibitors (PDE5Is) relax the urinary smooth muscle by increasing the cGMP concentration, finally improving LUTS. PDE5Is can significantly improve IPSS scores -2.8, but do not affect Q_{max} [117, 118]. Due to its prolonged halftime and bioavailability, allowing single administration per day, the PDE5 inhibitor tadalafil became available for medical treatment of voiding symptoms suggestive of BPH [37].

1.4.6 The role of plant extracts in LUTS therapy

Plant extracts have been used in the medical treatment of LUTS for many years. Some clinical studies from all parts of the world suggested that plant extracts could have some effects in relieving LUTS. However, the specific mechanism is not clear at all [119]. Many plant extracts are mixed preparations, containing a variety of ingredients from several plant species. It is still challenging to determine which ingredients play the predominant role or whether the different ingredients work together. Plant extracts containing phytoestrogens, fatty acids and lectins may have anti-inflammatory, antiandrogenic and estrogenic effects [120, 121]. It is believed that plant extracts can reduce prostate enlargement by inhibiting 5α-reductase activity [37]. In general, however, their mechanism of action and long-term efficacy are unclear.

In some Western countries, ethanol extracts of *Serenoa repens* have been used by LUTS patients for decades, despite lacking recommendation by treatment guidelines and doubtful effectiveness [37]. More recently, a meta-analysis reported that hexane extracts of *Serenoa repens* (Permixon®) have similar efficacy to tamsulosin and short-term 5-ARIs in relieving LUTS by reducing nocturia and improving Q_{max} compared with placebo [122]. Changes in outcomes showed a mean improvement with -6.06 points in IPSS, an increase of 2.29 mL/s in Q_{max}, and improved the life quality of the individuals [122]. The prostate volume decreased by -3.32 cm^3 to -5.37 cm^3, a mean reduction of approximately 6.8% [122].

In East Asian countries, plant extracts are used to treat LUTS as well. The most widely used extract is from “Gegen”, the dried root of *Pueraria* species from the *Fabaceae* family. Some previous studies reported that Gegen has a variety of biological activities, including antipathogenic, antihypertensive, antiplatelet, anti-inflammatory, and antiapoptotic [123, 124]. However, the effects of these plant extracts in non-traditional medicine and their mechanisms still require further investigation.

1.5 Role of non-adrenergic prostate/non-cholinergic bladder smooth muscle contraction

1.5.1 Non-adrenergic prostate smooth muscle contraction

The α_1-Adrenergic prostate smooth muscle contraction has been addressed earlier and by much more studies than the non-adrenergic prostate smooth muscle contraction, which was the focus of preclinical and clinical research for decades [125]. α_1-Blockers have been applied as the first-line medical treatment to improve LUTS by relaxing prostate smooth muscle tone [36]. However, cumulative evidence suggests that non-adrenergic agonists can also prostate smooth muscle contractions, notably with contractile forces being comparable to maximal prostate contractions by

α_1-adrenoceptors, which include in particular endothelin-1 and thromboxane A_2 (TXA_2) [36, 38, 126]. These non-adrenergic contractions will eventually cause prostatic urethral obstruction and may keep prostate smooth muscle tone and symptoms elevated despite treatment with α_1-blockers [127]. The awareness is increasing that the development of new strategies for future medications should address adrenergic plus non-adrenergic prostate smooth muscle contractions simultaneously [38].

Endothelin-1 is one of the endogenous endothelin peptides. The whole system also contains the G protein-coupled endothelin receptors, ET type A receptor and ET type B receptor, both activated by endothelin, whose activation will induce the elevation of intracellular free calcium, resulting in the contraction of the smooth muscles, including the smooth muscles in the urethral tract [128, 129].

Thromboxane A_2 is a prostaglandin. It has prothrombotic properties and was a known vasoconstrictor by activating thromboxane receptors. Thromboxane receptors are expressed on human prostate smooth muscle cells and thromboxane A_2 can induce prostate smooth muscle contraction [130]. The mechanisms of thromboxane A_2-induced prostate smooth muscle contraction may be related to Ca^{2+}/calmodulin-dependent mechanisms and Rho kinase. U46619, a synthetic thromboxane A_2 analogue, is a potent and stable thromboxane A_2 receptor agonist [130].

In total, adrenergic agonists and non-adrenergic agonists are involved in the contraction of prostate smooth muscle. In addition to endothelin-1 and thromboxane A_2, which induce strong contractions in human prostate tissues, further mediators have been proposed and cause contractions in prostate tissues from animals. However, most, if not all of them, do not induce relevant contractions in human tissues, so their possible role in the regulation of prostate smooth muscle tone may be limited to non-human prostates [38, 131].

1.5.2 Non-cholinergic bladder smooth muscle contraction

Cholinergic agonists always attracted the utmost attention in bladder smooth muscle contraction. Consequently, muscarinic receptor antagonists are applied for medical treatment in patients with OAB. However, analogue to the role of non-adrenergic agonists in the prostate, non-cholinergic agonists can also trigger the bladder detrusor contraction, in parallel to cholinergic contractions by muscarinic receptors. Thus, endothelin-1 and the thromboxane A_2 analogue U46619 have been confirmed to cause human bladder detrusor contraction [132, 133]. In fact, it was assumed that the spontaneous, strong detrusor contractions accounting for storage symptoms in OAB could be non-cholinergic and non-neurogenic, which may explain the limited efficacy of anticholinergics [134-136].

1.6 The β_3-adrenergic agonist mirabegron

1.6.1 Clinical use in OAB

The β_3-adrenoceptor primarily activates adenylate cyclase by coupling to $G\alpha_s$, subsequently promoting the conversion of ATP into cAMP, thereby rising intracellular levels of cAMP, finally resulting in the relaxation of smooth muscle [102, 137, 138]. Mirabegron is an agonist of β_3-adrenoceptor, with presumed high selectivity, and it is the first β_3-adrenoceptor agonist being approved for the treatment of OAB [107]. Before mirabegron became available, other β-adrenoceptor agonists (such as BRL-37344 and CGP-12177) were ineffective at the human receptor and failed in clinical applications [139, 140].

Following its introduction, clinical studies and experiences have confirmed that mirabegron has no higher efficacy than anticholinergic medications in reducing urinary incontinence and frequency of urination when it is used to treat OAB patients [77]. However, it does not have the same side effects as anticholinergic medications,

so it has a considerably lower incidence of self-discontinuation throughout the treatment period [111, 141].

1.6.2 Unresolved mechanisms underlying improvements of storage symptoms

Despite nearly ten years of clinical use of mirabegron, the mechanism underlying the improvements of storage symptoms by mirabegron is still unclear [113, 142, 143]. Mirabegron exhibits a high affinity for human β_3-adrenoceptors. Its binding constant for β_3-adrenoceptors is 2.5 nM [144], and maximum plasma levels during standard dosing amount to 137 nM in men [145]. These values are contrasted by the EC_{50} values required for relaxation of precontracted human detrusor tissues, ranging from 588 nM to 3.9 µM [146, 147]. This discrepancy makes it difficult to conclude that mirabegron exerts its clinically beneficial effects by acting on β_3-adrenoceptors in the detrusor smooth muscle cells of the bladder [148].

Previous in vitro studies using human bladder tissue focused on the relaxing effects of mirabegron on precontracted smooth muscle tissue. However, pre-contractions in these studies were weak and not in the range of full cholinergic detrusor contractions, which may be expected under physiological conditions during voiding. Obviously, only one study has examined the effects of mirabegron on full cholinergic contractions of human detrusor tissues, by constructing concentration-response curves for carbachol in the presence of 30 µM mirabegron (highly exceeding human plasma levels) and without mirabegron [102]. Thus, all previous studies have not conclusively illuminated the mechanisms of mirabegron effects in OAB. Central questions remain, and the initial concept that mirabegron improves storage symptoms by activation of β_3-adrenoceptors on bladder smooth muscle cells and by subsequent bladder smooth muscle relaxations has been challenged [148]. A proper understanding of these above seems advisable, which requires further comprehensive studies. Here, the concentration-dependent effects of mirabegron on

full neurogenic, cholinergic, and non-cholinergic contractions were investigated in human detrusor tissues.

1.6.3 Considerations for use in voiding symptoms suggestive of BPH

As it has been assumed that mirabegron may cause smooth muscle relaxation and considering that inhibition of prostate smooth muscle contraction is an important strategy for medical treatment of voiding symptoms in BPH, application of mirabegron in BPH in addition to OAB has been proposed. After the approval for OAB patients, however, clinical trials to verify the safety and efficacy of mirabegron have shown that it does not affect the Q_{max}, which means that mirabegron has only a very slight or no effect on voiding symptoms or on the urethral resistance due to the prostate [149]. In contrast, experimental studies reported that β_3-adrenoceptors are expressed in prostate tissue [52, 101]. In vitro studies using isolated human and non-human prostate tissues demonstrated that mirabegron can induce prostate smooth muscle relaxation [150, 151]. However, these studies were primarily based on rodent tissues [150]. Only one series of experiments used tissue from human beings, but used a mirabegron concentration of 10 µM [151], which is much higher than its K_i values for β_3-adrenoceptors (2.5 nM) [144], and its plasma levels during standard dosing in humans (137 nM) [145]. Evidence from experiments with non-human prostates and radioligand binding studies suggested antagonism of α_1-adrenoceptors by mirabegron [150]. Considering the role of α_1-adrenoceptors in pathophysiology and medical treatment of voiding symptoms suggestive of BPH, this off-target effect may be interesting or even promising [152]. However, it is not known, which concentrations of mirabegron are required for antagonism of α_1-adrenoceptors by mirabegron in human prostate tissues. Moreover, there are no data on non-adrenergic and neurogenic contractions of prostate tissues. Thus, although mirabegron has been used clinically in LUTS patients for several years, little is known about its role in prostate smooth muscle, and even the few available data remain inconclusive and raised questions. Here, the concentration-dependent effects of

mirabegron on full neurogenic, adrenergic, and non-adrenergic contractions were investigated in human prostate tissues.

1.7 Role of organ bath studies in experimental LUTS research

The organ bath is a device allowing to induce and to quantify smooth muscle contractions in isolated tissues in vitro. Contractions may be induced by the addition of endogenous agonists (e. g., α_1-adrenergic agonists, endothelin-1, U46619 to prostate tissues), or by stimulating action potentials in the nerves, by application of electric field stimulation (EFS) and resulting in contractile neurotransmission. Construction of concentration-response curves for agonists or of frequency response curves by EFS is possible, in the presence of drugs (e. g., mirabegron) and without drugs (controls) in the same experiment.

A typical organ bath device consists of several containers (“organ bath chambers”, four of them in the device used in this thesis), holding an oxygenated physiological buffer solution (“Krebs-Henseleit solution”) maintained at 37°C and an anchor point to hold the tissue, with a force sensor connecting the anchor point. The sensor can sense the force exerted to the anchor, and converts the force continuously into an electrical signal. So that force changes (=contractions) can be recorded and quantified. Even subtle changes that the naked eye cannot observe are finally quantified into conclusive data and to curves, through the calculation and recording of related software [153].

In organ baths, human or animal tissue samples can be used for physiological and pharmacological studies at the tissue level. Organ baths are currently widely used in research to evaluate the pharmacological effects of new drug candidates on the human body and can also explore the pathogenesis of certain diseases.

Increased prostate smooth muscle contraction is one of the leading causes of LUTS involved in BPH, and excessive activity of human bladder smooth muscle is a driving cause for LUTS in OAB. Testing the effects of different agonists, antagonists or inhibitors on these two kinds of smooth muscle tissue is the basis to understanding the pathophysiology of LUTS more deeply and finding new targets for future treatment options [154, 155].

2 Summary (English)

The term LUTS summarizes highly common disorders of the urinary tract, including voiding and post-voiding symptoms attributed to the prostate, and storage symptoms imparted by the bladder. Smooth muscle contractions are central for pathophysiology and treatment of LUTS, as increased prostate smooth muscle tone in BPH contributes to urethral compression and thus, to impaired bladder emptying and weak urinary flow, while spontaneous contractions in the bladder wall cause urgency, frequency and incontinence in OAB. Accordingly, inhibition of smooth muscle contraction by drugs is the most prominent strategy in medical treatment. However, the first-line options, i.e. α_1-blockers for voiding symptoms in BPH and anticholinergics for storage symptoms in OAB show limited efficacies and are afflicted by high discontinuation rates. For treatment of OAB, the β_3-adrenoceptor agonist mirabegron is available as alternative anticholinergics for more than 10 years.

Initially, and based on preclinical studies, it was assumed that the mechanism of mirabegron-induced improvements of storage symptoms is a relaxation of bladder smooth muscle, due to activation of β_3-adrenoceptors on bladder smooth muscle cells. Consequently, after the approval for OAB, it has been proposed that mirabegron may cause smooth muscle relaxation in the prostate as well and thereby improve voiding symptoms in BPH. Meanwhile, the concept that mirabegron

improves symptoms by bladder smooth muscle relaxation has been challenged, and the actions and use of mirabegron are afflicted by an increasing number of questions. Thus, the K_i value of mirabegron for β_3-adrenoceptors is 2.5 nM and maximum plasma levels amount to 137 nM, but EC_{50} values for mirabegron-induced relaxation of pre-contracted human bladder tissues amounted to 0.588-3.9 µM. Nearly all previous studies using human detrusor tissues focused on relaxation of pre-contracted tissues, but pre-contractions were unphysiologically low and thus, mirabegron effects were small. In contrast, the effects of full human detrusor contractions were only examined in one series of one study, but using a high concentration of 10 µM mirabegron. Moreover, effects on non-cholinergic contractions have never been reported, even though these may induce detrusor contractions as well, in parallel to cholinergic agonists and neurotransmission. Regarding human prostate tissues, previous data are limited to one series in one study, where 30 µM mirabegron was applied and inhibited α_1-adrenergic contractions. In contrast, mirabegron did not improve voiding symptoms suggestive of BPH in clinical trials.

The findings in the first publication of this thesis demonstrate that the effects of mirabegron on contractions of human prostate tissues are limited to off-target effects, which require concentrations in the micromolar range. Using a concentration of 1 µM, neither contractions by α_1-agonists nor neurogenic contractions induced by EFS were inhibited. Non-adrenergic contractions were even not inhibited by 10 µM mirabegron. Concentrations of 5 µM and 10 µM caused obvious right-shifts of concentration-response curves for α_1-adrenergic agonists and inhibited EFS-induced contractions, which was resistant to a β_3-adrenoceptor antagonist and strongly points to an off-target antagonism of α_1-adrenoceptors by mirabegron, if applied at micromolar concentrations. In conclusion and considering maximum plasma levels of 137 nM, an effect of mirabegron on voiding symptoms cannot be expected, even if “promising” off-target effects in vitro occur. In line with these findings, antagonism of

α_1-adrenoceptors by mirabegron and other β_3-antagonists has been suggested from other smooth-rich tissues.

The findings in the second publication of this thesis demonstrate that the effects of mirabegron on human detrusor tissues are limited to inhibition of neurogenic contractions, while contractions by cholinergic and non-cholinergic agonists are not affected at all, if full contractions are examined, instead of unphysiologically low pre-contractions as in most previous studies. Moreover, the inhibition of EFS-induced contractions required 10 µM mirabegron and was resistant to a β_3-adrenoceptor antagonist, but was not observed using 1 µM. Again, this may reflect an off-target effect, which inhibits the cholinergic neurotransmission in the bladder wall, but is unlikely, if not impossible to occur in vivo, in the face of maximum plasma levels of 137 nM. In conclusion, the amelioration of OAB storage symptoms by mirabegron is likely not attributed to activation of β_3-adrenoceptors in bladder smooth muscle cells. Rather, effects on afferent signals during bladder filling or on the central nervous system, which have been proposed as well, are more likely to be responsible for symptom improvements.

Together, these findings answer central questions around the use of mirabegron, which persisted despite its application for the treatment of LUTS for more than 10 years. Previously conflicting results were obviously attributed to unphysiological experimental conditions, leading to skewed conclusions.

3 Zusammenfassung (German)

Der Begriff LUTS (Symptome des unteren Harntraktes) fasst ausgesprochen häufige Störungen des Harntraktes zusammen. Diese umfassen Probleme mit dem Wasserlassen, die im Zusammenhang mit der Prostata stehen, sowie Speicherstörungen, die durch die Harnblase bedingt werden. Ein zentraler Faktor

sowohl in der Pathophysiologie als auch in der Behandlung dieser Symptome ist die glattmuskuläre Kontraktion. Bei einer BPH trägt ein erhöhter glattmuskulärer Tonus in der Prostata zu Verengungen der Harnröhre bei, und beeinträchtigt so die Blasenentleerung und schwächt den Harnstrahl ab. Bei einer überaktiven Blase (OAB) verursachen dagegen spontane Kontraktionen in der Blasenwand Harndrang und Inkontinenz. Dementsprechend ist die Hemmung der glattmuskulären Kontraktion eine wichtige Strategie in der medikamentösen Behandlung dieser Symptome. Allerdings zeigen auch die Optionen der ersten Wahl, also α_1-Blocker gegen Entleerungsstörungen bei einer BPH, ebenso wie Anticholinergika gegen Speicherstörungen einer OAB eine eingeschränkte Wirksamkeit, und sind zudem mit hohen Abbruchraten behaftet. Zur Behandlung der OAB steht seit über 10 Jahren der β_3-Adrenozeptor Agonist Mirabegron als Alternative zu Anticholinergika zur Verfügung.

Auf der Grundlage vorklinischer Studien wurde ursprünglich angenommen, dass die Verbesserungen von Speicherstörungen durch Mirabegron auf einer Relaxation der glatten Blasenmuskulatur beruhen, die auf eine Aktivierung von β_3-Adrenozeptoren durch Mirabegron hin erfolgen sollte. Folgerichtig wurde nach der Zulassung für die OAB-Behandlung spekuliert, dass Mirabegron auch in der Prostata zu glattmuskulären Relaxationen, und so zu Verbesserungen von Entleerungsstörungen bei BPH führen könnte. Mittlerweile wird das Konzept, dass Mirabegron durch eine glattmuskuläre Relaxation zu Verbesserungen von OAB-Symptomen führt, jedoch stark angezweifelt, und die Wirkungen von Mirabegron sind mit einer gestiegenen Zahl von Fragen und Unklarheiten behaftet, die sich wie folgt gestalten. Der K_i-Wert von Mirabegron für β_3-Adrenozeptoren beträgt 2.5 nM und die maximal erreichbaren Plasmakonzentration liegen bei 137 nM, wohingegen der EC_{50} für Mirabegron-induzierte Relaxationen von vorkontrahierten humanen Blasengeweben zwischen 0,588-3,9 µM liegt. Nahezu alle früheren Studien an humanen Detrusor-Geweben untersuchten die Effekte auf vorkontrahierte Gewebe, allerdings

waren diese Vorkontraktionen unphysiologisch niedrig und die Effekte von Mirabegron auf den Muskeltonus dementsprechend gering. Die Effekte auf vollständige Detrusor-Kontraktionen, die bei der Miktion erfolgen, wurden dagegen lediglich in einer Versuchs-Serie einer einzigen Studie untersucht, wobei jedoch eine hohe Konzentration von 10 µM Mirabegron verwendet wurde. Darüberhinaus wurden die Effekte auf nicht-cholinerge Kontraktionen nie berichtet, obowohl diese ebenfalls Detrusor-Kontraktionen auslösen, parallel zu cholinergen bzw. neurogenen Kontraktionen. Frühere Untersuchungen an humanen Prostata-Geweben sind auf eine Versuchs-Serie in nur einer Studie beschränkt, die mit 30 µM Mirabegron durchgeführt wurde, was zu einer Hemmung α_1-adrenerger Kontraktionen führte. Im Gegensatz dazu wurde in klinischen Studien keine Verbesserung von BPH-bedingten Entleerungsstörungen beobachtet.

Die Ergebnisse der ersten hier vorgestellten Publikation zeigen, dass sich die Effekte von Mirabegron auf Kontrakionen humaner Prostata-Gewebe auf off-target-Effekte beschränkt, welche Konzentrationen im mikromolaren Bereich erfordern. Eine Konzentration von 1 µM führte zu keinerlei Hemmung der α_1-adrenergen oder neurogenen, EFS-induzierten Kontraktionen. Nicht-adrenerge Kontraktionen wurden selbst durch 10 µM Mirabegron nicht gehemmt. Konzentrationen von 5 µM und 10 µM verursachten deutliche Rechts-Verschiebungen von Konzentrations-Wirkungs-Kurven für α_1-adrenerge Agonisten und hemmten EFS-induzierte Kontraktionen, was sich als resistent gegen einen β_3-Adrenozeptor-Antagonist erwies. Dies weist stark auf eine Antagonisierung von α_1-Adrenozeptoren durch Mirabegron hin, also auf einen unspezifischen Effekt, der bei mikromolaren Konzentrationen von Mirabegron auftritt. Dementsprechend und in Anbetracht von maximalen Plasmakonzentrationen von 137 nM, kann ein Effekt von Mirabegron auf Entleerungsstörungen nicht erwartet werden, selbst wenn in vitro “vielversprechende” off-target”-Effekte zu beobachten sind. Analog zu den hier erzielten Ergebnissen legten Ergebnisse anderer Studien einen Antagonismus von

α_1-Adrenozeptoren durch Mirabegron und andere β_3-adrenerge Agonisten in anderen glattmuskulären Geweben nahe.

Die Ergebnisse der zweiten hier vorgestellten Publikation zeigen, dass sich die Effekte von Mirabegron auf humane Detrusor-Gewebe auf eine Hemmung neurogener Kontraktionen beschränken, während Kontraktionen durch cholinerge und nicht-cholinerge Agonisten nicht gehemmt werden, wenn vollständige Kontraktionen untersucht werden, anstatt niedrige Vorkontraktionen wie in den meisten früheren Studien. Darüberhinaus erfordert die Hemmung neurogener Kontraktionen 10 µM Mirabegron und erwies sich als resistent gegen einen β_3-Adrenozeptor Antagonisten, wohingegen 1 µM ohne Effekt blieb. Dies spiegelt abermals einen off-target-Effekt wieder, der zu einer Hemmung der cholinergen Neurotransmission in der Blasenwand führt, aber in Anbetracht von maximalen Plasmakonzentrationen von 137 nM mit sehr großer Wahrscheinlichkeit nicht in vivo auftritt. Dementsprechend ist die Verbesserung von Speicherstörungen der OAB durch Mirabegron höchstwahrscheinlilch nicht auf eine Aktivierung von β_3-Adrenozeptoren in glatten Muskelzellen des Detrusors zurückzuführen. Vielmehr dürften den klinischen Effekten Wirkungen auf afferente Signale während der Blasenfüllung, oder auf das Zentralnervensystem zu Grunde liegen, was ebenfalls in anderen Studien vorgeschlagen wurde.

Die hier vorgestellten Ergebnisse beantworten zentrale Fragen rund um den Gebrauch von Mirabegron und im Zusammenhang mit seinen Wirkungen, die trotz seiner bereits über 10-jährigen klinischen Anwendung bestanden. Frühere, widersprüchliche Ergebnisse waren offenbar auf unphysiologische Versuchsbedingungen zurückzuführen, was zu verzerrten Schlussfolgerungen führte.

4 Publicaion I

Title: Concentration-dependent alpha1-Adrenoceptor Antagonism and Inhibition of Neurogenic Smooth Muscle Contraction by Mirabegron in the Human Prostate.

Journal: Frontiers in Pharmacology.

Author: Ru Huang, Yuhan Liu, Anna Ciotkowska, Alexander Tamalunas, Raphaela Waidelich, Frank Strittmatter, Christian G Stief, Martin Hennenberg.

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5 Publication II

Title: Inhibition of Full Smooth Muscle Contraction in Isolated Human Detrusor Tissues by Mirabegron Is Limited to Off-Target Inhibition of Neurogenic Contractions.

Journal: Journal of Pharmacology and Experimental Therapeutics.

Author: Ru Huang, Alexander Tamalunas, Raphaela Waidelich, Frank Strittmatter, Christian G Stief, Martin Hennenberg.

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