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Effects of the integrin inhibitors BTT3033 and BOP, and the integrin-linked kinase inhibitor Cpd22 on human prostate smooth muscle contraction

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1 Introduction

1.1 Anatomy and histology of the prostate

The prostate is a male sexual organ, which is a chestnut-shaped gland located directly below the apex of the bladder (as shown in figure 1), with the urethra passing through inside, and seminal vesicles accompanying outside above the back in humans [1-3].

In primates, the prostate surrounds parts of the urethra. The segment of the urethra surrounded by the prostate is termed the prostatic urethra or the proximal urethra. A pair of ejaculatory ducts passes through the prostate and has an opening in the prostatic urethra along with the prostatic ducts (as shown in figure 1) [2, 3].

The prostate can be divided into the anterior fibromuscular stroma and three glandular zones: the central zone, the transition zone and the peripheral zone. In the normal state, the peripheral zone constitutes 70% of glandular prostate in mass, while the central zone constitues 25% and the

transition zone 5%. There is a fibromuscular band surrounding the prostate, with the term "prostate capsule" referring to it [3, 4].

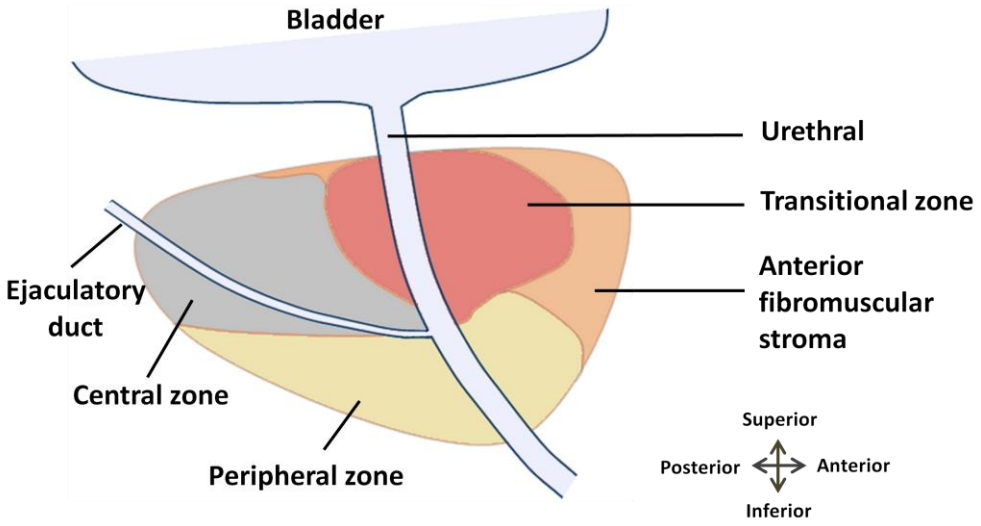

Figure 1. Sagittal plane of human prostate. The prostate is located below the bladder. The urethra and the urine flow run through the prostate. The ejaculatory ducts pass through the prostate and open in the prostatic urethra (The figure is adapted from "Sathianathen, N. J., Konety, B. R., Crook, J., Saad, F., & Lawrentschuk, N. (2018). Landmarks in prostate cancer. Nature Reviews Urology.").

The glandular prostate contains ducts and acini, which are similar in cell constitution and morphological pattern. Among the cells that form ducts and acini, glandular epithelial cells are androgen-receptor-rich secretive cells which synthesize prostatic fluid [5]. Basal cells are not as distinguishable morphologically or immunohistochemically as other cells,

and are considered to haver differentiation potential [2, 5]. Endocrine -paracrine cells are not well known, and can only be shown by ancillary techniques. They are assumed to mediate growth and function of the prostate [5].

The stroma lies between the glands, contributing to about half of the prostate volume. The fibromuscular stroma consists of abundant smooth muscle cells distributed randomly in direction surrounding the glands, and is separated by elastic fibers [2].

1.2 Physiology of the prostate

1.2.1 Function

As an exocrine gland, the prostate secretes the milky fluid called prostatic fluid, serving to nourish and protect sperm. The prostate is an organ for balanced production, storage, composition, and pulse-triggered release of its product, owing to its substantial architecture containing acini and smooth muscle. The prostatic fluid is extremely abundant in Zn^{2+}, citrate and kallikreins [6]. The concentration of citrate in the prostatic fluid is about 1000 fold as in plasma, while zinc ranges about 500 fold [7, 8].

Accumulation of Zn^{2+} in prostatic fluid is achieved by Zn^{2+} transporters, which makes the prostate as the organ or body compartment with the highest Zn^{2+} concentration. [7]. Citrate is crucial for adenosine 5'-triphosphate (ATP) formation of sperm [9], while Zn^{2+} can facilitate citrate formation. Zn^{2+}, citrate and kallikreins together participate in promoting fertility by controlling the molecular function of the sperm in the ejaculatory process like liquefication and motility [8].

For healthy men, the prostate smooth muscle contracts during the ejaculatory process to press the stored prostatic fluid out into the urethra, while in patients with benign prostatic hyperplasia (BPH), the smooth muscle tone increases and contributes to dynamic obstruction of the bladder outlet [10]. In normal conditions, the fibromuscular band can confront the pressure from the urethra. In morbid conditions, the band restricts carcinoma from invasion and metastasis, but it promotes the mechanical obstruction by facilitating the pressure increase to the urethra in the prostate when the prostate gets enlarged [2, 4, 11, 12].

1.2.2 Hormonal regulation and nerve innervation

The development and growth of the prostate are regulated by androgen through the androgen receptor. As an androgen, 5α-dihydrotestosterone

(DHT) is synthesized from testosterone by 5α-reductase in the prostate, and it is strictly essential for prostate formation and development from the early beginning since the embryonic period [13]. Lack of DHT causes prostate absence or dysplasia. Under the continuous effect of DHT, the prostate grows and transforms into the adult phenotype by 21-30 years old [14, 15]. Then, the prostate continues to grow slowly starting at the normal average weight of 20 g. When men come to an age of 51-60 years old, 50 percent have an enlarged prostate weighing 33 g on average [14]

The secretory function is also highly dependent on DHT, and without DHT the prostatic function is expected to get lost. Ageing is paralleled by reduced testosterone and also intraprostatic DHT, which damages the ability of prostate epithelial cells to concentrate Zn^{2+}, and to synthesize secretive citrate and kallikreins, finally compromising fertility [8]. Other hormones also have roles in regulating the secretion function. Citrate production, for instance, receives regulation by DHT, prolactin [7] and estrogens [6].

Other evidence shows that the growth and secretion of the prostate can also be regulated neurogenicallly. The prostate is mainly innervated with autonomic nerves. It has been found that sympathetic nerves mediate

growth via α_1 and β_2 adrenergic receptors, while cholinergic [16] and β-adrenergic nerves innervate glandular epithelial parts with the potential to mediate secretion [17] [18].

One of the main effects of the nerve innervation is to mediate smooth muscle contraction and relaxation. Prostatic smooth muscle receives massive noradrenergic innervation, which mainly mediate contraction [19]. Nitric oxide (NO) and parasympathetic nerves are responsible for smooth muscle relaxation [18] [20]. Acetylcholine, NO and neurotransmitters like adenine nucleotides and nucleosides, opioids, neuropeptide Y (NPY) and vasoactive intestinal peptide [21] may act as co-transmitters or modulators in autonomic neurotransmission supplying prostate stroma and smooth muscle tone [19].

Apart from adrenergic neurotransmission, some hormonal or other non-adrenergic agonists including endothelins and thromboxane A2 (TXA2) induce prostate smooth muscle contraction, and contribute to prostate smooth muscle tone [22, 23].

1.3 Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is a result of hyperplastic growth of the stroma and glandular epithelium, and occurs most obviously in the transition zone [24]. Technically, BPH is only the histologic condition able to result in benign prostatic enlargement (BPE) [25]. Bladder outlet obstruction (BOO) is the clinical condition, which is often a result of benign prostatic obstruction (BPO) caused by BPE, considering the urine flow from the bladder going through the prostatic urethra [26]. BOO can be aggravated by the dynamic factor resulting from increased tone of prostate smooth muscle [10].

1.3.1 Epidemiology and etiology

BPH is related to ageing. Until an age of 25–30 years, the prostate enlarges to a "normal" weight of about 20 g. From this age, prostate growth is stopped or only slow, until age-related BPH is beginning. Thus, the Olmstead County Study reported longitudinal data suggesting an annual prostate growth rate of 1.6 % as measured by transurethral ultrasonography [27]. Symptomatic BPH usually emerged after 40 years. The prevalence of BPH is 25 % among men 40 to 49 years of age, 50 % among men of 50 to

60 years old, and more than 80 % among men of 70 to 79 years old [27]. DHT was assumed to be the continuous stimulating factor resulting in BPH progression related to aging, with the risk of BPH for men who have the highest DHT level nearly 3-fold higher than men with lowest levels [28]. However, recent research reported that estrogen also contributes the growth of prostate when men get aged [6].

Other studies have also demonstrated that genetic factors may also be involved in BPH. A study took the youngest quartile (< 64 years old) with a resected prostate greater than 37 cm^3 from patients who were under prostatectomies for BPH as probands, and found the risk of brothers of the probands is 6 times higher than the controls [29]. Familial BPH patients have been found to had larger prostate volume and normal serum androgen levels, which indicated that the genetic factor may be involved in prostate growth independently from androgen [30]. Ethnicity differences may further explain the genetic factor. Black men and Asian men were found to be less likely to have BPH than white [31, 32]. Caucasian from southern Europe have the highest risk [31].

Apart from those unchangeable or inherited factors, some risk factors can be modified through lifestyle changes. Obesity, for instance, has been

revealed to profoundly increase the risk of BPH [27]. It was shown that physical exercise decreased the risks of BPH profoundly [33, 34], and an unhealthy diet contributed to BPH in multiple patterns [35].

Various studies showed that systemic diseases can affect the blood supply of the prostate. Theses diseases are related to BPH, and include cardiovascular diseases like systemic atherosclerosis and metabolic diseases like diabetes [36, 37]. The pathophysiology behind it may concern increased growth factors under the hypoxic condition [38]. Studies also revealed that metabolic diseases can cause autonomic nerve disorder and contribute to prostate growth via corresponding receptors [39]. In patients with metabolic syndrome, related regulatory molecules can directly result in BPH [40, 41].

Local conditions like prostatic inflammation are also considered to be a factor for BPH development. Under consistent stimulation of inflammatory cytokines, epithelial cells are driven to grow in the chronic inflammation condition [42].

1.3.2 Complications

BPH is related to clinically relevant complications, including urinary retention, haematuria, urinary tract infection, bladder calculi, hydronephrosis, incontinence [3, 43, 44]. Complications are important indexes for BPH progression and likely indications for surgery [3, 43-45].

Urinary retention (UR) consists of acute UR (AUR), chronic UR (CUR) or acute on chronic UR. AUR has a sudden onset and could be extremely painful [46], while CUR is usually without pain and featured by increased residual urine after voiding, which can usually palpable or percussible. AUR may also present in CUR patients [47].

Haematuria usually results from the friable hypervascularity in BPH [48]. Medication for BPH like finasteride may decrease angiogenesis and improve haematuria [48]. Haematuria also results from infection and calculi.

Urinary tract infection (UTI) and bladder calculi occur when BPH patients can not sufficiently empty the bladder. This provides an environment for bacteria or calculi growth. Recurrent UTI and bladder calculi are absolute indications for surgery [49].

Hydronephrosis is usually secondary to CUR in BPH, since patients with CUR have significantly higher bladder pressure and may finally affect the pressure in the renal pelvis resulting in hydronephrosis [49]. Chronic renal failure is the final and severest prognosis in this situation, so hydronephrosis is also an absolute indication for surgical intervention [3, 49].

Incontinence may be due to overactive detrusor independent from or secondary to long-term BOO and CUR in BPH patients, called urgency incontinence. Overflow incontinence may also present in BPH patients with CUR [50].

1.4 Male lower urinary tract symptoms

Lower urinary tract symptoms (LUTS) are a group of symptoms caused by various diseases, classified as voiding symptoms and storage symptoms [3, 43]. Voiding symptoms include “hesitation, difficulty, intermittence and weakness of urination, and terminal dribbling”, while storage symptoms consist of “urgency, frequency, incontinence and nocturia” [3].

1.4.1 Causes

Causes of male LUTS are quite widespread. Problems concerning bladder function or its outlet may be the causes (showed in figure 2) [45]. Apart from the disease in the lower urinary system, the causes can also be non-urological diseases affecting the whole body system, which may include cardiovascular or respiratory system, nervous system, endocrine system, as well as any other conditions that would facilitate inflammation, cause hyperplasia of detrusor or prostate, damage the function of nerve innervation, or influence contraction of detrusor or the bladder outlet-related smooth muscle including prostate smooth muscle. For aged men, BPH is the most common cause of LUTS [44].

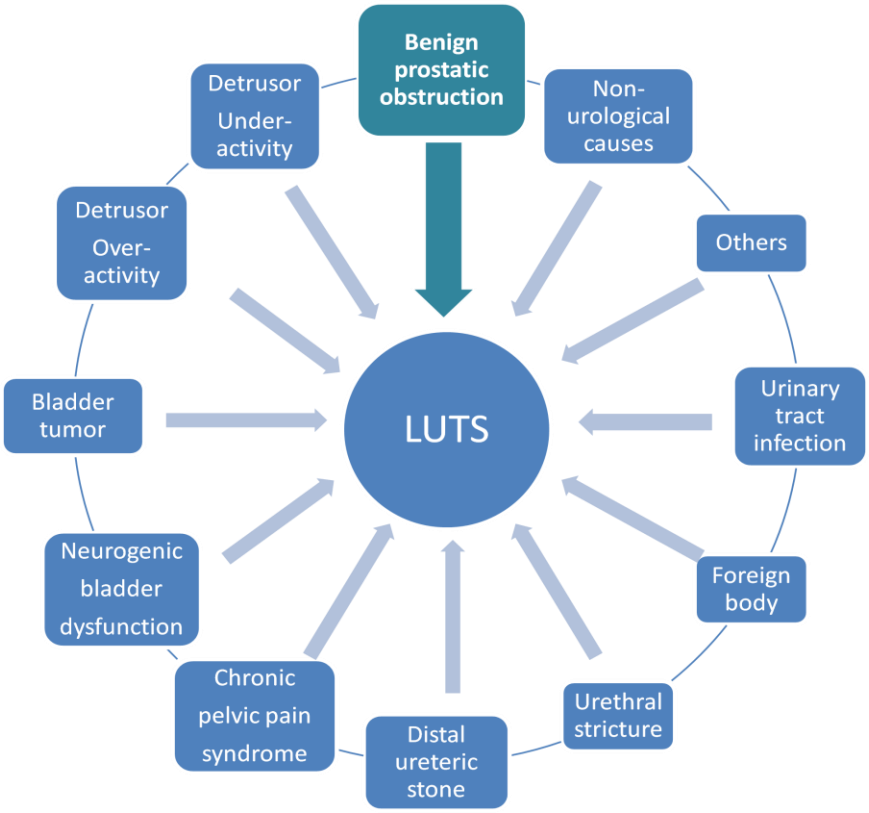

Figure 2. Causes of male LUTS. Benign prostatic obstruction is the most common cause of male LUTS, other urological and non-urological causes contribute as well. (The figure is adapted from Oelke M, Bachmann A, Descazeaud A, Emberton M, Gravas S, Michel MC, et al. (2013). EAU guidelines on the treatment and follow-up of non-neurogenic male lower urinary tract symptoms including benign prostatic obstruction. European urology.")

1.4.2 Burden

LUTS are common in both genders especially for people older than 40 years. For males, as described already above, BPH is the most important

reason for LUTS. According to statistics, LUTS affected 2.3 billion people worldwide in 2018, and nearly half of them were men [51]. LUTS are especially highly prevalent in aged men, with 70% of men ≥ 80 years reported LUTS and 51.6% of them reported moderate-to-severe LUTS [52, 53]. BPH alone costs more than 180 million pounds each year in the United Kingdom [54] and 1.1 billion dollars in the single year of 2000 in the United States [55]. Among these costs, around 60% are spent for management of BPH complications. Quality of life (QOL) in male LUTS patients has been decreased due to bothersome symptoms and the fact that LUTS patients are also related to anxiety, depression, and unsatisfied sexual life. QOL of partners of male LUTS patients has been compromised as well [27].

1.4.3 The role of BPH in LUTS

Enlargement of the prostate due to BPH causes increased resistance to urinary flow, so that mechanical obstruction of the bladder outlet due to increased prostate size contributes to voiding symptoms and to increased postvoid urine volume [3, 45]. Although BPH mainly starts in the transition zone and the degree of compression on the prostatic urethra is the most crucial, total prostate size is still an important index. Researches concerning the relationship between prostate size and LUTS show that

men with a prostate volume of 40.1-80 ml are more likely to develop bothersome and progressive LUTS in the future even with mild or no LUTS than men with a prostate volume below 40 ml. Larger prostates correlated with increased risk of acute urinary retention and are more likely to progress to surgery [49].

Enlarged prostates can weaken urine flow by compressing the urethra, which causes voiding (obstructive) symptoms, consisting of hesitancy, weakness, straining, prolonged micturition, incomplete emptying, dribbling, etc [44, 45]. At the early stage of BPH, the detrusor gets stronger and the bladder wall gets hyperplasic to compensate for the obstruction of the bladder outlet. Eventually, the detrusor loses the ability to confront the pressure, and then becomes weak and unable to empty itself, which is called decompensation. Increased postvoid urine volume may result in detrusor hyperplasia, which in turn may cause storage (irritative) symptoms including frequency, urgency, urge incontinence and nocturia [3, 44]. An enlarged prostate can also directly compress the triangle district in the bladder, contributing to the irritative symptom [49]. If the condition continues to progress, the patient will suffer from urinary incontinence or other complications like hydronephrosis. What should be pointed out is that BPH causes bladder obstruction by not only mechanical compression to the urethra, but also dynamic obstruction of the bladder outlet owing to

increased prostate smooth muscle tone [27].

1.4.4 Current medical treatment of male LUTS

The current medicational treatment of male LUTS [44] follows the strategy illustrated in figure 3.

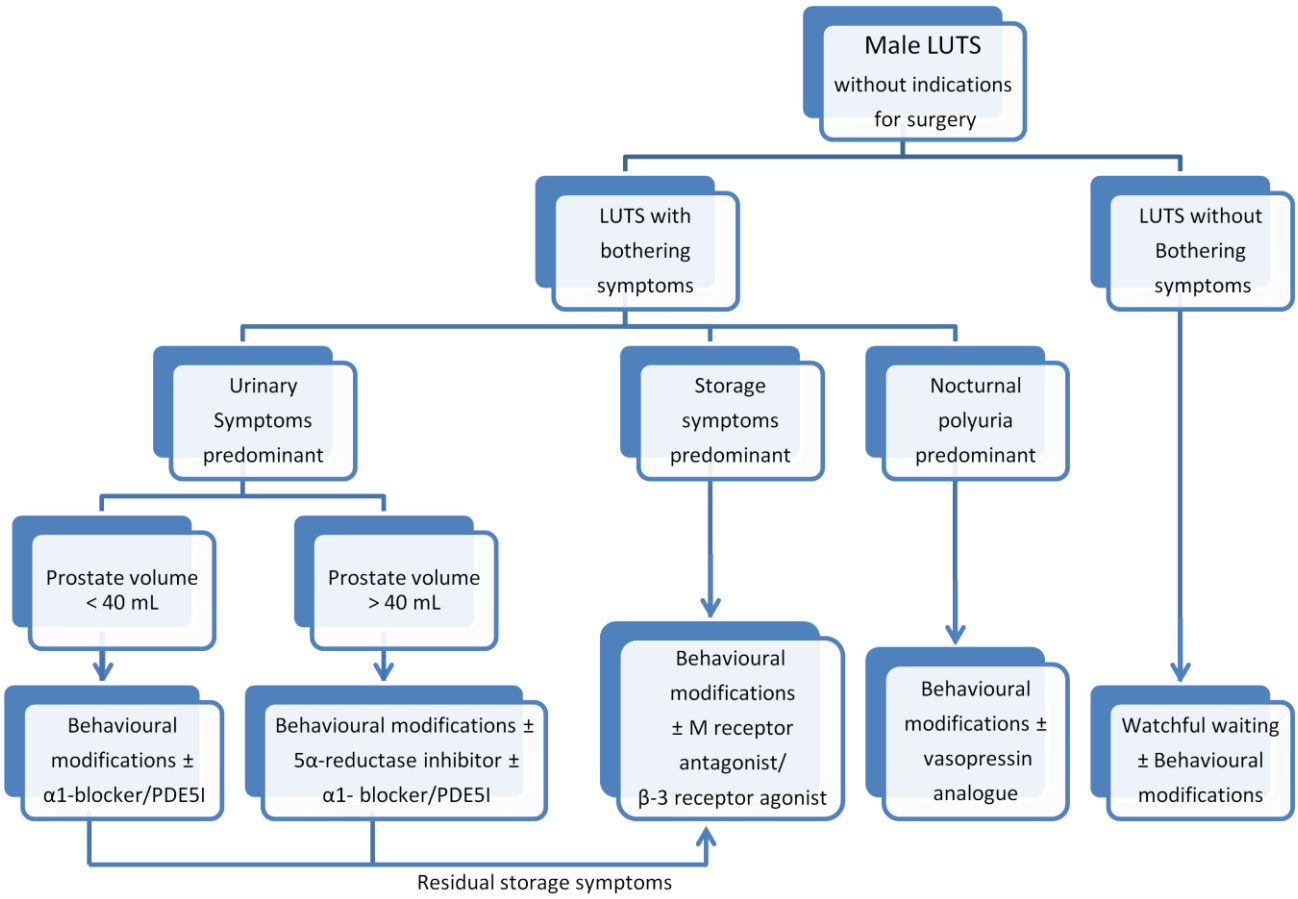

Figure 3. Non-surgical treatment algorithm for male LUTS. The algorithm includes different LUTS occuring in men, i. e. obstructive („urinary“) symptoms suggestive of BPH, and bladder-related symptoms including storage symptoms and nocturia. The depicted procedures are based on the guidelines of the European Association of Urology (EAU) for treatment of male LUTS [44].

Alpha$_1$-adrenoceptor blockers

Alpha$_1$-(α_1)-adrenergic receptors mediate prostate smooth contraction and contribute to basic tension of prostate smooth muscle. They are responsible for smooth muscle resistance of the urethra to urinary flow, and

consequently represent important determinants of bladder outlet obstruction. Thus, α_1-blockers can theoretically relieve the resistance of the bladder outlet. Alpha$_1$-selective blockers include tamsulosin, alfuzosin, terazosin, doxazosin and silodosin. Researches suggest that α_1-blockers can decrease International Prostate Symptom Score (IPSS) and improve quality of life [56], and objectively improve urinary voiding function, i.e. the maximum flow rate (Q_{max}) [57]. Researches found that the effect of the α_{1a}-adrenoceptors subtype predominates in the contraction of prostate smooth muscle [58]. Tamsulosin is the most commonly used medication for LUTS suggestive of BPH [59], and the selectivity of tamsulosin for the α_{1a} subtype is tenfold higher than for the α_{1b} subtype [60], while silodosin is selectively targeting on α_{1a}. Clinical studies suggested that silodosin has advantages in relieving nocturia and lowering side effects outside the lower urinary tract compared to other α_1-blockers. However, silodosin shows a higher risk of ejaculatory dysfunction [61].

5α-reductase inhibitors

5α-reductase inhibitors (5-ARIs) inhibit the conversion of testosterone to DHT. DHT has a higher affinity to androgen receptors than testosterone. Considering that the proliferation of prostate cells is relying on androgen, 5-ARIs can change the natural progression of the BPH process [62]. 5-ARIs focus on the mechanical obstruction resulting from BPE. Unlike

α_1-blockers, they can decrease the acute urinary retention rate [63]. Side effects may include erectile dysfunction, decreased sex drive or retrograde ejaculation [64].

Phosphodiesterase (PDE) inhibitors

Currently, PDEs inhibitors are widely used in erectile dysfunction, which mainly target on PDE5 who degrade [65] guanosine 3', 5'-cyclic monophosphate (cGMP) and consequently relaxes the vascular smooth muscle controlling the blood flow of the corpus cavernosum. Recently, a PDE5 inhibitor has been approved for treatment of LUTS suggestive of BPH, regardless of the presence or absence of ED. PDE5 inhibitors have been shown to reduce prostate smooth contractions [66] and improve IPSS [67]. In fact, however, PDE5 inhibitors have limited effects in LUTS (i. e. resembling those of α_1-blockers), and combination therapy with α_1-blockers is more effective than monotherapy with PDE5 inhibitors [68, 69].

Vasopressin analogue

If the patient shows LUTS mainly about nocturnal polyuria, vasopressin analogues can be used to reduce the amount of urine produced during the night. Desmopressin is the most commonly used vasopressin analogue.

Vasopressin analogues can only be applied for men under 65 years to avoid side effects like hyponatremia [70].

Muscarinic receptor antagonists

Muscarinic receptor antagonists are available to treat overactive bladder considering that contraction of the detrusor is driven by muscarinic receptors[71]. They are recommended to treat storage symptoms of men with LUTS suggestive of BPH combined or without combination with medications reducing outlet obstruction [44]. Although there is a theoretical concern that muscarinic receptor antagonists may increase residual urine volume and acute urinary retention rate, clinical trials show relief in storage symptoms without obvious effects on voiding symptoms or AUR rate [39, 44, 72]. Although solifenacin is a selective antagonist for the subtype 3 muscarinic receptor (M_3), discontinuation due to side effects such as dry mouth could be the critical limit of antimuscarinics. Sometimes a combination of antimuscarinics and salivary-gland-specific muscarinic agonists are needed to reduce dry mouth [44, 59].

β_3-adrenoceptor agonists (β_3-agonists)

Similar to muscarinic receptor antagonists and considering the distribution

and function of β_3-adrenoceptors in the bladder, its agonists are assumed to improve OAB by relaxation of detrusor smooth muscle. Mirabegron is best known β_3-adrenoceptor agonist used for LUTS treatment. One of the advantages of β_3-agonists is to reduce the side effect of antimuscarinics. It has been revealed that the combination treatment of β_3-agonists and half-dose antimuscarinics significantly reduced side effects compared with only using antimuscarinics in full dose [73].

Limitations of current medications

Among the medical options above, α_1-adrenoceptor antagonists are still the most important and commonly used, as they directly target prostate smooth muscle contraction by inhibiting α_1-adrenergic receptors, and relieve the compression of prostatic urethra [60, 74]. Alpha$_1$-blockers reduce the IPSS by 30-40% and increases of Q_{max} of 20-25% [75]. However, placebos cause improvements of IPSS by up to 34%, and increase Q_{max} by up to 27% [76]. Moreover, α_1-blockers do not change the prostate volume, with limited evidence to prevent AUR or other severe complications [74]. Up to 69 % of patients may not be satisfied by treatment with α_1-blockers, thus, the efficacy is not satisfying enough, considering the age-dependency of prevalence [22, 23, 77, 78]. Using 5-ARIs solely or combined with α_1-blockers can not only improve IPSS and Q_{max}, but also reduces the prostate volume and risk of AUR. However, 36-45 % of patients are still

unsatisfied even using a combination therapy of α_1-blockers with 5-ARIs [79]. Side effects and unsatifying efficacy contribute together to the discontinuation rate of combination therapy [45].

Non-adrenergic mediators induce prostate smooth muscle contraction apart from α_1-adrenceptors and include receptors like thromboxane A2 receptors and endothelin receptors contributes as well, apart from α_1-adrenoceptors. The magnitude of those non-adrenergic contractions is comparable to adrenergic contractions, and was shown to be sufficient to replace α_1-adrenergic tension in the human prostate in ex vivo experiments [23]. However, non-adrenergic receptors and the contractions mediated by them are beyond α_1-blockers' reach. It is assumed that the non-adrenergic contraction of prostate smooth muscle is the reason why the efficacy is limited since the treatments with α_1-blockers ignore this component [22].

Other current options like PDE5 inhibitors, which cover non-adrenergic contractions are either less efficient as α_1-blockers in relaxing prostate smooth muscle, or as parts of combination therapy complimentary to α_1-blockers [80]. Although combination therapies includes 2 or 3 medications are promising to compliment each other, it should be considered that additional medications also bring about discontination rate

and potential additive side effects [45].

More importantly, apart from the current understanding of prostate smooth muscle contraction, the whole picture of prostate smooth muscle contraction may be much broader. Recently, various new concepts have been indicated, which may also explain why current medical therapy is not efficient enough [80].

1.5 Mechanisms of prostate smooth muscle contraction and relaxation

Although the smooth muscle is equipped with different receptor subtypes in different organs, the mechanisms of smooth muscle contraction/relaxation in different body systems have a lot in common. The common base of regulation is that the phosphorylation level of myosin light chain (MLC) determines the contractile state of smooth muscle, since MLC phosphorylation enhances myosin-actin filament cross-bridge attachment and promotes cycling of actomyosin [81]. In a word, MLC phosphorylation results in contraction, while MLC dephosphorylation brings about relaxation [81]. In addition, actin polymerization and tissue integrity are ultimate prerequisites of smooth muscle contraction. All these

mechnisms are regulated and induced by several intracellular signaling pathways. The currently well established models of the regulations are described in the following paragraphs.

1.5.1 Contraction

The current understanding of intracellular mechanisms mediating smooth muscle contraction is summarized in figure 4. Like striated muscles, smooth muscle contraction can be mediated by increases of the cytosolic Ca^{2+} concentration [82, 83]. However, the extent of MLC phosphorylation or force of contraction does not necessarily parallel the intracellular Ca^{2+} concentration [81]. This points to other mechanisms, and Ca^{2+}-independent pathways of contraction regulation have also been recognized. Ca^{2+}-dependent pathways start with agonist-induced activation of corresponding receptors, followed by activation of phospholipase C (PLC) by receptor-coupled G proteins. PLC then hydrolyzes phosphatidylinositol diphosphate (bound in the cell membrane) into diacylglycerol [84] and inositol-1,4,5-trisphosphate (IP3). IP3 triggers Ca^{2+} outflow from the sarcoplasmic reticulum into the cytosol, which is followed by depolarization of the smooth muscle cell and opening of voltage-gated Ca^{2+} channel [82, 83]. An influx of Ca^{2+} causes a further elevation of cytosolic Ca^{2+} which binds to and activates calmodulin (CaM)

which subsequently activates MLC kinase (MLCK), resulting in MLC phosphorylation and force generation [81, 83, 85, 86]. In parallel, DAG increases the activity of protein kinase C (PKC) which inhibits MLC phosphatase (MLCP), resulting in inhibition of MCL dephosphorylation, and promotion of contraction. The receptor-coupled G protein also activates a Ca^{2+}-independent pathway by activating guanine nucleotide exchange factors (GEFs). GEFs then activate the small GTPase RhoA and its effector Rho kinase (ROCK1/2), which can inhibit MLCP and promote contraction [81, 85, 86].

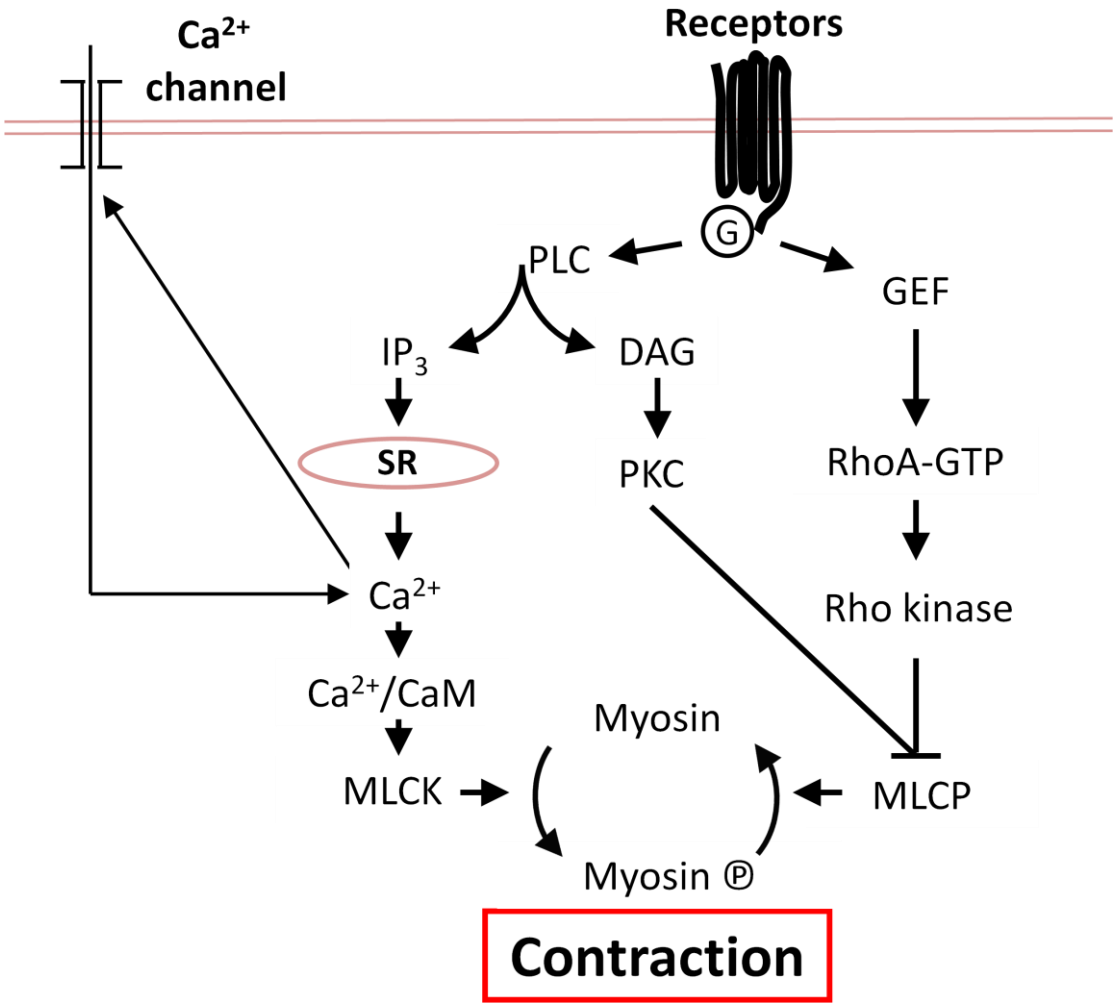

Figure 4. Mechanisms of smooth muscle contraction. The contraction of smooth muscle is a result of MLC phosphorylation, which can be achieved by MLCK activation and MLCP inactivation, under different pathways, dependent or independent of Ca^{2+}.

1.5.2 Relaxation

Relaxation relies on active regulations through cyclooxygenase [87], NO [20] or autonomic-nerve neuroendocrine pathway. Adenosine 3',5'-cyclic monophosphate (cAMP, cyclic AMP) and cGMP are second messengers

mediating smooth muscle relaxation. The cAMP is catalyzed by adenylyl cyclases (AC), which are activated downstream by β-adrenoceptors or receptors binded by COX-produced prostaglandins (PGs) [85], while cGMP is catalyzed by soluble guanylyl cyclase (sGC) which is activated by NO [20]. Cyclic AMP and cGMP activates protein kinase A [88] and protein kinase G (PKG), respectively. PKA and PKG facilitate K^+ inflow which promotes Ca^{2+} exchange ATPase on the sarcoplasmic reticular or the plasma membrane, and directly activate MLCP resulting in dephosphorylation of MLC and relaxation (as shown in figure 5) [81].

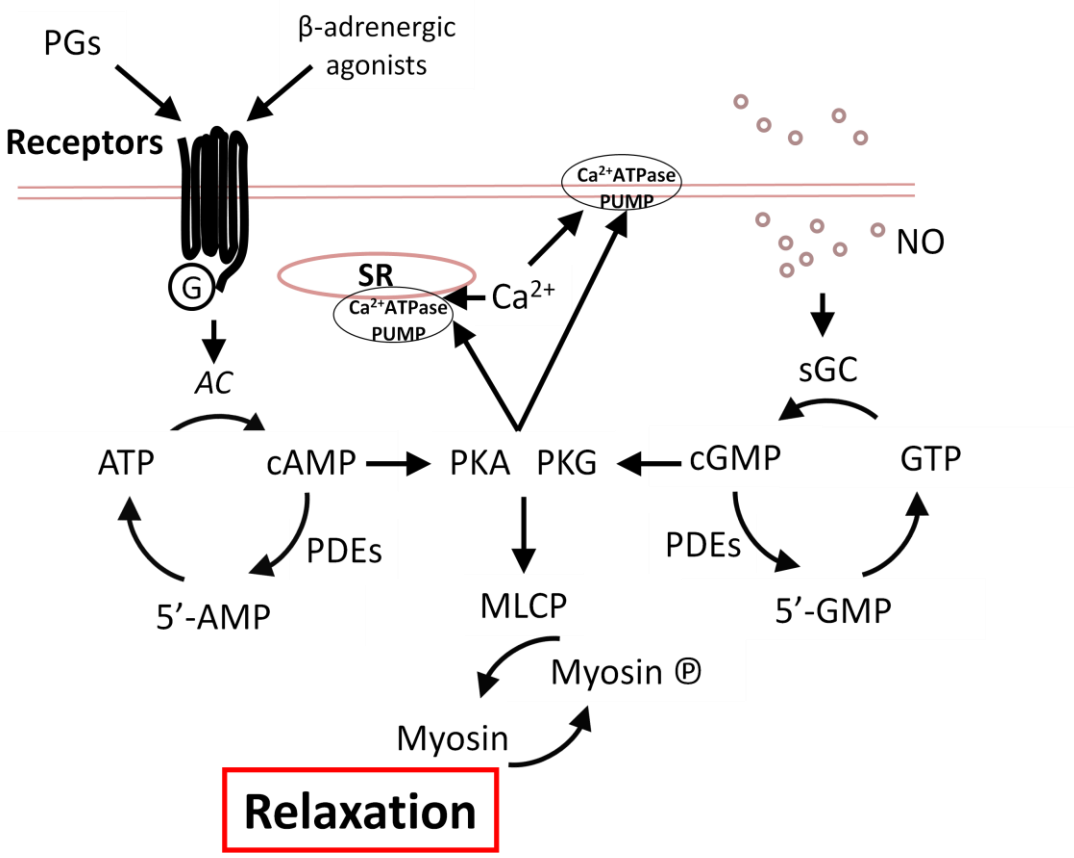

Figure 5. Regulatory mechanisms of smooth muscle relaxation. The relaxation of smooth muscle relies on MLC dephosphorylation, which can be mediated by PKA and PKG, downstream of pro-relaxation receptors or NO.

1.6 Novel targets for inhibition of prostate smooth muscle contraction

Considering the high prevalence among aged men, as well as the fact that the efficacy and the patient compliance of available medications are still

insufficient, novel pharmacological options for medical treatment of LUTS suggestive of BPH are of high demand. In recent years, various studies have been performed to explore novel targets for potential treatment options for LUTS suggestive of BPH. These studies either identified novel targets in the classical pathways shown above, or extended the understanding of prostate smooth muscle contractions, indicating new candidate compounds.

1.6.1 Neurotransmission

Neurotransmitters mediate neurogenic prostate smooth muscle contraction through transmembrane receptors [81]. Thus, inhibition of neurotransmission is supposed to attenuate the contraction, what can potentially improve LUTS suggested of BPH. It has been shown that electric field stimulations (EFS)-induced contractions can be enhanced by a thiol-alkylating agent N-Ethylmaleimide (NEM), and are inhibited by the adrenergic neuron blocker guanethidine [89]. A recent study reported that FRAX486 and IPA3, inhibitors of P21-activated kinase (PAK) can inhibit contractions of prostate smooth muscle induced by EFS, but not those by noradrenaline and phenylephrine. Considering different PAK isoforms colocalized with tyrosine hydroxylase (a marker for catecholaminergic nerves) and the fact that PAKs are related to neuron function, it is assumed

that PAKs might mediate prostate smooth muscle by affecting on adrenergic neurotransmission [90].

1.6.2 Novel membrane receptor antagonists

G protein-coupled receptors and protein kinases and represent two prominent pathways for cellular signaling. Traditional receptor antagonists are described above and include mainly adrenergic and cholinergic ligands. Novel receptor antagonists either provide higher selectivity for the traditional targets or address new targets.

AdTx1, for instance, is an α_{1a}-adrenoceptor antagonist which may be highly selective for prostate or bladder neck, and it can efficiently inhibit adrenergic contraction of prostate smooth muscle [65, 91].

The thromboxane A2 receptor antagonist picotamide showed inhibition effect of TXA2- and endothelin-mediated contraction. Surprisingly, the α1-adrenergic and neurogenic contractions were also reported to be inhibited in the human prostate [78, 92].

Previous studies have indicated that not only noradrenaline but also ATP is

secreted from the sympathetic synapse in the prostate after EFS [93]. Studies showed that P2X-receptors (ATP receptors) indeed emerge in the prostate smooth muscle [93]. P2X-receptor antagonists may therefore relieve urethral compression from the prostate [93].

Epithelial cannabinoid receptor type 1 (CB1) receptors showed the inhibition effect of neurogenic prostate smooth muscle contraction of animal models [94].

1.6.3 Protein kinases

Protein kinases are crucial mediators in intracellular signaling, among which serine/threonine kinases and protein tyrosine kinases (PTKs) are the most important ones. PTKs and some serine/threonine kinases like mitogen-activated protein kinase (MAPKs) can mediate cell proliferation and differentiation, with the potential not only to reduce contraction but also to inhibit the growth of the prostate [95, 96].

A recent study of the group of the author of this thesis suggested that an inhibitor for serine/threonine kinase 16 (STK16) inhibited non-adrenergic and non-neurogenic human prostate smooth muscle contractions,

suggesting that STK16 may play a role in a non-adrenergic signal pathway in addition to current established pathways [97].

Notably, apart from inhibiting prejunctional transmission shown by inhibition of EFS-induced contraction of prostate smooth muscle, the PAK inhibitors FRAX486 and IPA3 also inhibited endothelin-1 induced contraction [90]. This suggested that PAK, as an serine/threonine kinase may be involved not only in the transmission of sympathetic nerve but also in a non-neurogenic signal pathway. Inhibitors for LIM kinase, SR7826 and LIMKi3, have been previously shown to inhibit EFS, adrenergic and non-adrenergic contractions of human prostate strips [98]. The c-Jun N-terminal kinase inhibitors SP600125 and BI-78D3 reduced agonist-induced adrenergic human prostate smooth muscle contractions, with no non-adrenergic contractions were tested [99]. Polo-like kinase (PLK) may also be engaged in the prostate smooth muscle contraction, as its inhibitors were able to inhibit α_1-adrenergic contractions of human prostate tissues, but not the non-adrenergic contractions of them [100]. These findings indicated that different serine/threonine kinases may play roles in the prostate smooth muscle contraction through α_1-adrenergic, non-adrenergic mediators or both.

Non-receptor PTKs are widely involved in crucial cell functions including proliferation and differentiation, with potentials not only to reduce contraction but also to inhibit growth of prostate. Src family kinases (SFKs), ZAP70 family, Tec family, JAK family and intranuclear PTK are among the non-receptor PTK. A previous study revealed that two inhibitors of SFKs, AZM475271 and PP2, can not only inhibit adrenergic and neurogenic contraction of human prostate smooth muscle but also proliferation of WPMY-1 cells (prostate stromal cell lines). Proliferation reduction could not be seen in c-Src-deficiency cells [101]. Focal adhesion kinase (FAK), a non-receptor PTK linking integrin to intracellular signaling pathways, has also been observed to play a role in contractile responses in the prostate, with FAK inhibitors being able to reduce adrenergic and neurogenic smooth muscle contractions of the human prostate [102].

Recently, some anti-proliferation compounds designed for inhibiting tumor growth have also been shown to reduce prostate smooth muscle tone and to inhibit the growth of prostate cells at once. Thus, onvansertib, as a selective PLK1 inhibitor, was designed to inhibit tumor growth including prostate cancer. Recently, it was shown to be able to reduce neurogenic, adrenergic, non-adrenergic and purinergic contractions of human prostate strips, and to reduce the proliferation of WPMY-1 cells [103]. Imatinib

mesylate (Gleevec) as a PTK inhibitor, has also been shown to facilitate the relaxation of human prostate strips [104].

1.6.4 Small monomeric GTPase dependent pathways

Among small GTPases, the Ras superfamily is the most important group, which consists of five families Rho, Ras, Rab, Arf, and Ran [105]. Rho has GDP/GTP-binding activity and GTPase activity, and can be activated when GDP–Rho is converted to GTP–Rho by GEFs, downstream of receptor-coupled $G\alpha_{12/13}$. The activity of Rho kinases can then be enhanced by binding GTP–RhoA. Rho kinase includes ROCK1 and ROCK2, which inhibits MLCP and promote contraction [81, 106]. In mammals, the family of Rho GTPases consists of Rho (isoforms A–E, and G), Rac, Cdc42 and TC10. RhoA, Rac1 and Cdc42 have been the most intensively investigated in smooth muscle [86, 107]. The ROCK1/2 inhibitor Y27632 and the ROCK-2-selective inhibitor fasudil have been reported to inhibit adrenergic contraction of human prostate smooth muscle [108]. Y27632 has also been found to inhibit non-adrenergic contractions of the human prostate smooth muscle [109]. The Rac inhibitors NSC23766 (a selective inhibitor of Rac1-GEF interaction) and EHT1864 (a potent inhibitor for Rac family) have been found to inhibit neurogenic and adrenergic contraction of the human prostate smooth muscle [110]. According to

another recent study using human prostate strips, EHT1864 also inhibits endothelin-1- and U46619-induced contractions, and NSC23766 inhibits U46619-induced contraction [22]. Apart from this, both inhibitors show inhibition of growth of prostate stromal cells, so that a potential to target both dynamic and mechanical compression of prostatic urethral in BPH patients with LUTS was concluded [110]. ARF6 is another small monomeric GTPase. Cytohesins, belonging to GEFs, may activate ARFs directly [41]. The Sec7 inhibitor H3 (secinH3), which is a cytohesin-specific inhibitor, was reported to inhibit ARF6, but not RhoA or Rac1. In parallel, secinH3 has been revealed to inhibit neurogenic, adrenergic and non-adrenergic contractions of human prostate smooth muscle [111]. NAV2729, an inhibitor for ARF6, has also been found to inhibit EFS-induced and adrenergic contractions of human prostate strips [41]. These findings indicate that ARF6 may also be involved in prostate smooth muscle contraction.

Cyclic AMP and cGMP are second messengers mediating smooth muscle relaxation. Cyclic AMP is produced by AC, downstream of β-adrenoceptors or COX-produced PGs [81, 106, 112], while cGMP is formed by soluble guanylyl cyclase (sGC) which is activated by NO [20]. Cyclic AMP and cGMP activate PKA [88] and PKG respectively, as well as exchange proteins directly activated by cAMP (EPAC), Epac, Rap1,

Tiam1, Vav2, Rac, etc to mediate smooth muscle relaxation [111]. Direct stimulation of cAMP and cGMP was also reported to be able to reverse ET-1-induced smooth muscle contraction from the human prostate [113]. PDE inhibitors prevent PDEs from degrading cAMP and cGMP, thus promote relaxation of the prostate smooth muscle. Apart from PDE5 inhibitors which are already approved for current therapy of LUTS suggestive of BPH, the PDE10-selective inhibitor TC-E 5005 has also been reported to inhibit mainly adrenergic, neurogenic human prostate smooth muscle contractions [114]. On the other hand, the cAMP effector EPAC may be involved in prostate relaxation, as its activators pCPT and OME were able to inhibit phenylephrine-induced human prostate smooth muscle contraction under addition of cyclooxygenase inhibitor indomethacin. KMUP-1, as an sGC activator and PDE5 inhibitor, has been observed to facilitate the accumulation of cAMP/cGMP and to upregulate expression of sGC, PKG, and PKA protein in rat prostate, as well as to inhibit the prostate smooth muscle contraction induced by phenylephrine [115].

1.6.5 Plant extracts

Isosamidin, an extract of Peucedanum japonicum was reported to attenuate contractions induced by phenylephrine of the human prostate strips [116].

Similar effects were also observed by Honokiol, a constituent of Magnolia species, with its ability to inhibit adrenergic contraction of human prostate strips, apart from which, it also facilitated apoptosis of stromal cells [117]. According to research using rat tissues, Serenoa repens, a liposterolic extract of saw palmetto has been found to significantly reduce prostate smooth muscle contractile responses via a non-specific mechanism [118].

1.6.6 Targets of metabolic syndrome

Metabolic syndrome comprises disorders like hypertension, atherosclerosis, obesity, and insulin resistance, and atherogenic dyslipidemia, caused by genetic and acquired factors [41, 119]. Recently, the relationship between LUTS and metabolic syndrome has been shown by clinical data. In patients with metabolic syndrome, adipokines play a central role for pathophysiology [41]. Ghrelin is one of such adipokines. At preclinical level, it has been reported that ghrelin may promote prostate enlargement, growth of prostate cells, and prostate smooth muscle tone [41]. Combination therapy with omega-3 fatty acids plus tamsulosin and finasteride has been used in the treatment of men with LUTS and BPH in a clinical research, and reported better clinical results [120].

1.7 Integrins and integrin-linked kinase

Integrins are transmembrane proteins widely expressed on the surface of various cells in different organs. Integrins are heterodimers which always consist of two subunits: α and β. In mammals, there are eighteen α and eight β subunits of integrins, and they assemble a total of 24 acknowledged integrins through non-covalent bonds between α and β [121, 122]. Integrins mediate adhesion and intercommunication between cells as well as between cells and extracellular matrix. Extracellular ligands for integrins outer domains embrace fibronectin, vitronectin, collagen, and laminin, while cytoplasmic domains of integrins are linked to the actin cytoskeleton as well as signaling molecules [121, 122]. Through these integrin-related signaling molecules, signals go across the membrane and regulate cell function, proliferation, differentiation, migration, morphology, mechanical homeostasis, as well as tissue regeneration and their integrity. Among these signaling molecules, kinases like integrin-linked kinase (ILK), FAK and Src family are found crucial. FAK and Src family have been revealed to effect on smooth muscle contraction of human prostate tissue [101, 102].

$Beta_1$ integrins connect the cells with the extracellular matrix. In vivo

studies showed that the inhibition or conditional deletion of β_1 integrins can inhibit smooth muscle in cardiovascular and respiratory systems [123, 124]. However, the roles of integrins and ILK in smooth muscle contractions have never been investigated in the prostate. In current studies concerning smooth muscle in the lower urinary tract system, only integrin α_v was shown to mediate contractility of human bladder smooth muscle cells in vitro [102].

Thus, based on integrin-functions in organs and cell types outside the lower urinary tract, and considering the recently suggested role of Src family kinases and FAKs in prostate smooth muscle [101, 102, 121], a role of integrins and ILK for regulation of prostate smooth muscle contraction could be assumed.

2 Objective of this thesis

Prostate smooth muscle contraction accounts for dynamic urethral obstruction in LUTS suggestive of BPH. Thus, inhibiting prostate smooth muscle is one of the most important strategies in the medical treatment. However, the current medicational therapies have their respective limitations.

To effectively reduce prostate tone, the ideal therapy should consider not only adrenergic but also non-adrenergic contraction. Thus, if one compound can have a different or wider spectum than α_1-blockers, i.e. also affect non-adrenergic contractions, it would be attractive. Recently, several compounds have been found to potently inhibit non-adrenergic contractions with or without affecting the adrenergic contractions of the prostate smooth muscle [90, 97]. Some compounds showed such effect, however, there is no evidence showing that the effects are confined in the prostate or the lower urinary tract. Non-selective effects may bring about side effects outside the lower urinary tract, which contribute to discontinuation. Thus, targets with multiple functional isoforms and different organ-specific pattern of distrubution may be a promising

direction for developing new medications. Integrins are assembled by various α and β subunits and distributed heterogeneously in different organs. In this study, the $\alpha_2\beta_1$ inhibitor BTT3033, the $\alpha_4\beta_1/\alpha_9\beta_1$ inhibitor BOP, and the ILK inhibitor Cpd22 were to tested to find out possible roles of these targets in prostate smooth muscle contraction, which has never been reported. Therefore the aim of this thesis was to address the following questions:

(1) Are integrins targeted by BTT3033 and BOP and ILK expressed in the human prostate?

(2) Are they expressed in smooth muscle cells or in glandular cells of the human prostate?

(3) Does BTT3033 inhibit neurogenic, non-adrenergic, or α_1-adrenoceptor-induced contractions of human prostate strips?

(4) Does BOP inhibit neurogenic, non-adrenergic, or α_1-adrenoceptor-induced contractions of human prostate strips?

(5) Does Cpd22 inhibit neurogenic, non-adrenergic, or α_1-adrenoceptor-induced contractions of human prostate strips?

3 Materials and methods

3.1 Reagents and devices

Table 1. Reagents used in this study.

Products	Manufacturer
Potassium chloride (KCl)	Roth, Germany
Sodium chloride (NaCl)	Roth, Germany
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Roth, Germany
Sodium bicarbonate ($NaHCO_3$)	Roth, Germany
D(+)-Glucose	Roth, Germany
Milk powder	Roth, Germany
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Sigma-Aldrich, USA
Potassium hydrogen phosphate (KH_2PO_4)	Applichem, Germany
L-Norepinephrine hydrochloride	Sigma-Aldrich, USA
(R)-(−)-Phenylephrine hydrochloride	Sigma-Aldrich, USA
Methoxamine hydrochloride	Sigma-Aldrich, USA
Custodiol® HTK cardioplegia	Dr. Franz Köhler Chemie, Germany
U46619	Enzo, USA
Endothelin-1	Enzo, USA
Cpd22	Merck, Darmstadt, Germany

BOP	Tocris, UK
BTT3033	Tocris, UK
Dimethyl sulfoxide (DMSO)	Roth, Germany
Rabbit anti-integrin α_2b (#13807)	Cell Signaling Technology, USA
Rabbit anti-integrin α_4 (#4600)	Cell Signaling Technology, USA
Rabbit anti-integrin α_9 (PA5-27771)	Invitrogen, Carlsbad, CA, USA
Mouse anti-integrin β_1 (TA807138)	Origene, Rockville, MD, SA
Rabbit anti-integrin β_1 (#35784)	Signalway Antibody, College Park, MD, USA
Rabbit anti ILK1 (#3862)	Cell Signaling Technology, USA
Mouse monoclonal anti-pan-cytokeratin (sc-8018)	Santa Cruz Biotechnology, USA
Mouse monoclonal anti-calponin 1/2/3 (sc-136987)	Santa Cruz Biotechnology, USA
Mouse monoclonal anti-PSA (sc-7316)	Santa Cruz Biotechnology, USA
Mouse monoclonal anti-β-actin (sc-47778)	Santa Cruz Biotechnology, USA
Biotinylated horse anti-mouse IgG	Vector Laboratories, CA, USA
Biotinylated goat anti-rabbit IgG	Vector Laboratories, CA, USA
Chemiluminescence ECL Hyperfilm	GE Healthcare, Germany
Cy3-conjugated goat anti-mouse IgG	Millipore, USA
4’,6’-diamidino-2-phenylindole-dihydrochloride (DAPI)	Invitrogen, Camarillo, CA, USA
Bovine serum albumin	Gibco, USA
Cy5-conjugated goat anti-rabbit IgG	Millipore, USA
10X Reaction Buffer	Thermo Fisher Scientific, USA
Film developers	Kodak, USA

Table 2: Devices used in this study.

Devices	Manufacturer
Tissue Bath System-720MO	Danish Myotechnology, Denmark
Tissue Bath System-720M	Danish Myotechnology, Denmark
Electrical Stimulator-CS4	Danish Myotechnology, Denmark
Water bath	Memmert, Bayern, Germany
Lab pump	KNF- Neuberger, USA
Vibration shaker-Vortex 3	IKA, Staufen, Germany
Protran® nitrocellulose membranes	Schleicher & Schuell, Germany
Gel electrophoresis system	Bio-rad, Germany
Superfrost® microscope slides	Thermo Fisher, USA
Laser scanning microscope	Leica SP2, Germany

3.2 Human prostate tissues

Human prostate tissue from a total of n=173 patients were obtained from radical prostatectomy for prostate cancer performed at the Department of Urology, Klinikum Großhadern, Ludwig-Maximilians University. Tissues from patients who previously had transurethral resection of the prostate (TURP) or brachytherapy were excluded. The tissues were transported to the Department of Pathology from the operation room immediately after the protates were removed from the patients, within Custodiol® cardioplegia organ-protection fluid with HTK

(histidine-tryptophan-ketoglutarate). Prostate samples were prepared by the pathologists within 30 minutes after the prostate was detached from each patient. The prostate was cut along the front middle line longitudinally until the urethra, and a sample was cut from the periurethral zone through the section surface, where smooth muscle is abundant and the risk of tumor is low. Tissues were tumor-free and showed no macroscopical signs of inflammation, as checked by experienced pathologists.

The tissues were transported to the laboratory in Custodiol® fluid, and cut into 4 smaller strips before being spiked on the needles in the organ bath. The exceptions were the prostate tissue designed to be used in Western blotting or immunofluorescence experiments, which were transferred to -80 ℃ after being shock frozen in liquid nitrogen. The whole process took less than 90 minutes after the removal of the prostate from the patient to the start of the organ experiment or the accomplishment of storage at -80 ℃.

3.3 Western blotting

- Under Custodiol, prostate tissue samples of about 0.3 x 0.1 x 0.1

cm were cut from prostate tissues. After siphoning off residual Custodiol, the pieces were shock-frozen with liquid nitrogen and moved to -80 ℃ for storage.

- Frozen prostate tissues were homogenized in a buffer containing 25 mM Tris/HCl, 10 µM phenylmethanesulfonyl fluoride, 1 mM benzamidine, and 10 µg/ml leupeptin hemisulfate, using the FastPrep®-24 system with matrix A (MP Biomedicals, Illkirch, France).
- The homogenates were centrifuged at 20,00g for 4 min, and the supernatants were collected.
- Aliquots of supernatants were assayed for protein concentration using the Dc-Assay kit (Biorad, Munich, Germany)
- In parallel, remaining the supernatant was boiled for 10 min with sodium dodecyl sulfate (SDS) sample buffer (Roth, Karlsruhe, Germany).
- Samples (20 µg/lane) were subjected to SDS-polyacrylamide gel electrophoresis.
- The proteins were electroblotted on Protran® nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany).
- Membranes were blocked with phosphate-buffered saline (PBS) containing 5 % milk powder (Roth, Karlsruhe, Germany)

overnight.

- The membranes were then incubated with rabbit anti-integrin α_2b (#13807) (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-integrin α_4 (#4600) (Cell Signaling Technology), rabbit anti-integrin α_9 (PA5-27771) (Invitrogen, Carlsbad, CA USA), mouse anti integrin β_1 (TA807138) (Origene, Rockville, MD, USA), rabbit anti ILK1 (#3862) (Cell Signaling Technology), mouse monoclonal anti-pan-cytokeratin (sc-8018), mouse monoclonal anti calponin 1/2/3 (sc-136987), mouse monoclonal anti-PSA (sc-7316), or mouse monoclonal anti-β-actin antibody (sc-47778) (if not other stated, from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 ℃ overnight. All primary antibodies were diluted in PBS containing 0.1 % Tween 20 (PBS-T) and 5 % milk powder.
- The membranes were then washed with PBS-T three times, with five minutes each time.
- Then, the membranes were incubated with secondary biotinylated horse anti-mouse or horse anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA), followed by incubation with avidin and biotinylated horseradish peroxidase (HRP) from the "Vectastain ABC kit" (Vector Laboratories, Burlingame, CA, USA) both diluted 1:200 in PBS. Incubation took 1 h at room temperature.

- Membranes were washed three times every five minutes with PBS-T after incubation with secondary antibodies or biotin-HRP.
- Membranes were washed with PBS once.
- Blots were developed with enhanced chemiluminescence (ECL) using ECL Hyperfilm (GE Healthcare, Freiburg, Germany).

3.4 Fluorescence staining

- Human prostate tissues were embedded in optimal cutting temperature (OCT) compound, then snap-frozen in liquid nitrogen and transfered to -80 ℃ for storage.
- Sections (8 µm) were cut in a cryostat and collected on Superfrost® microscope slides. The slides were washed with PBS.
- Sections were post-fixed in methanol at -20 ℃ and blocked in 1 % bovine serum albumin for 30 min.
- Sections were then incubated with primary antibody overnight, at room temperature all the time during the incubation.
- For double labeling, the following primary antibodies were used: rabbit anti-integrin α_2b (#13807) (Cell Signaling Technology,

Danvers, MA, USA), rabbit anti-integrin α_4 (#4600) (Cell Signaling Technology), rabbit anti-integrin α_9 (PA5-27771) (Invitrogen, Carlsbad, CA USA), rabbit anti-integrin β1 (#35784) (Signalway Antibody, College Park, MD, USA), rabbit anti-ILK1 (#3862) (Cell Signaling Technology), mouse monoclonal anti-pan-cytokeratin (sc-8018) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or mouse monoclonal anti-calponin 1/2/3 (sc-136987) (Santa Cruz Biotechnology, Santa Cruz).

- Control groups were stained without any primary antibody.
- The sections were then washed with PBS-T three times, with five minutes each time.
- Binding sites were visualized using biotinylated Cy3-conjugated goat anti-mouse IgG (AP124C) (Millipore, Billerica, MA, US) and Cy5-conjugated goat anti-rabbit IgG (Millipore, Billerica, MA, US).
- The sections were then washed with PBS-T three times, with two minutes each time.
- Nuclei were counterstained with 4’,6’-diamidino-2-phenylindole-dihydrochloride (DAPI) (Invitrogen, Camarillo, CA, USA).
- The sections were then rinsed in PBS-T three times with two minutes

intervals.

- The sections were dehydrated with 95% ethanol for two min, and then 100% ethanol twice with three minutes each time.
- Anti-fade mounting medium was applied.
- Immunolabeled sections were analyzed using a laser scanning microscope (Leica SP2, Wetzlar, Germany). Fluorescence was recorded with separate detectors.

3.5 Prostate tissue contraction measurements

- Preparation of Krebs–Henseleit (KH) solution and its two sub solutions: the compositions of sub solutions KH1 and KH2 are described in table 4 below. As table 4 shows, 43.5 ml of KH1 and 1.62 g glucose were added in 1000ml double distilled (D-D) water. Carbogen (95 % O_2, 5% CO_2) was continuously introduced into the solution for at least 20 min, before 43.5 ml of KH2 was added. The KH solution was always kept at 37 ℃ and under continuous carbogen (95% O_2 and 5% CO_2) inflow (as shown in figure 6).

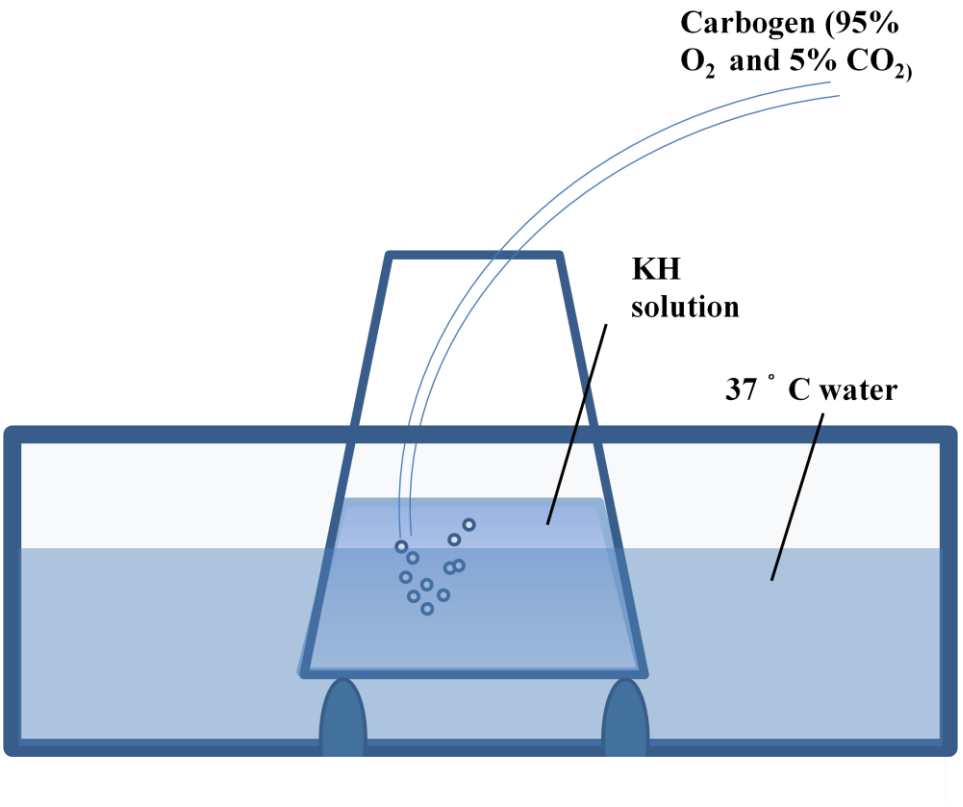

Figure 6. The KH solution in a water bath and under carbogen inflow.

The KH solution was kept in 37 ℃ thermostatic water bath and inserted with a tube with continuous carbogen flow to be on standby for use.

Table 3: Composition of solutions used in organ baths.

Solution	Component	
Krebs-Henseleit solution in organ bath (Guirgis et al.)	KH1	43.5ml
	Glucose	1.62g
	KH2	43.5ml
	Distilled water	1000ml
Presolution 1 (KH1)	NaCl	172.4g
	KCl	8.8g
	$CaCl_2 \cdot 2H_2O$	9.2g
	KH_2PO_4	4.1g
	$MgSO_4 \cdot 7H_2O$	7.4g
	Distilled water	add to 1000ml
Presolution 2 (KH2)	$NaHCO_3$	32.5g
	Distilled water	add to 1000ml

- Pretension: Each chamber was filled with 10 ml KH solution after washing three times with KH solution. The organ bath chambers were under continuous carbogen inflow and a stabilized temperature of 37 ℃, which was maintained by the setting of the myograph system. Strips of prostate tissue were cut into 4 pieces of approximately 0.6 × 0.3 × 0.3 cm as homogenically as possible, which were then spiked on the paired needles in the chambers by a needle distance of 0.4 cm (as shown in figure 7). The tensions of the

prostate tissues were adjusted with the aim that the tensions were stable at 4.9mN. In the pretension process, usually, at least three stretches maximal to 6.0 mN (the intervals were 15 min) were required for rebalancing, considering the tissue tends to relax before having stabilized during equilibration at 4.9 +/- 0.1 mN in minimally 45 min.

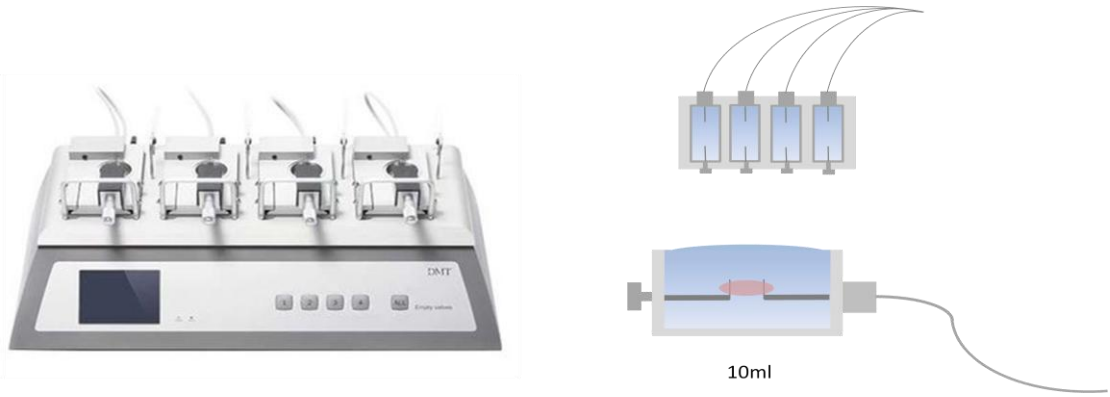

Figure 7. The myograph machine and strips in the organ bath chambers. A myograph device contains 4 chambers. Each chamber has a capacity of 10 ml liquid, with a independent adjustment knob and a force transducer.

- Reference contraction of prostate strips: KCl-induced contraction of prostate strips was induced by addition of 400 µL of 2 M KCl solution to reach a final KCl concentration of 80 mM. After the contraction reached the peak, the chamber was washed by KH solution 3 times.

- Incubation: BTT3033, Cpd22 and BOP were dissolved in DMSO, and 10 µl of BTT3033 (1 mM), Cpd22 (3 mM) or BOP (1 mM) were added to chambers, which were regarded as experiment group, with the final concentrations of 1 µM for BTT3033, 3 µM for Cpd22 and 1 µM for BOP. Correspondingly, 10 µl of DMSO was added in chambers which were regarded as controls. Incubation of inhibitors or solvent took 30 min before the contraction induction by EFS or agonists was started.
- EFS application or agonist addition: EFS was applied in doubling increasing frequences from 2Hz to 32Hz. Addition of agonists was conducted as shown in table 5 to achieve cumulative concentrations of 0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM (not used in ET-1), 30 µM (not used in ET-1) and 100 µM (not used in U46619 and ET-1).
- Temperature setting and force calibration: The temperature setting of myograph system and the flask water bath were adjusted to keep the KH solution in the chambers or flask 37 ℃ according to the accessory probe or kerosene thermometer.
- Calibration of the force transducer was performed with an 1:1 L-shaped right-angle lever, until the force output showed 9.81+/-0.1 mN after a two-gram standard weight was placed on the vertical part of the lever. Temperature settings and force calibrations were done every three months or every time after relocation of the machine or a

replacement of force transducer.

3.6 Data analysis and statistics

Data collected from the contraction experiments are presented as means ± standard deviation (SD) [127]. One-way analysis of variance [128] [128] was used to compare the whole response curves, and multi-variance analysis was used to compare contractions at each concentrations. P values <0.05 were considered significant. This study and analyses were designed to be exploratory, so p values are considered as descriptive. Groups (control and inhibitor group) have identical group size if being compared. No data or experiments were excluded from analyses.

4 Results

4.1 Detection of integrins, ILK, PSA, calponin and cytokeratin in human prostate tissues by Western blotting

Seven human prostate tissues were subjected to Western blotting for detection of integrin subunits α_2b, α_9, α_4, β_1, as well as ILK1, PSA, calponin, pan-cytokeratin, and β-actin using corresponding antibodies. PSA can serve as an indicator for the BPH degree, calponin as a maker for smooth muscle content and pan-cytokeratin as a maker for glandular epithelial cells [97]. Beta-actin is a housekeeping protein used as a loading control. Detection of all antigens revealed bands with sizes matching the expected molecular weight of the corresponding proteins. These presumed bands for all related integrin subunit and ILK1 occurred using all included prostate tissues, whereas the intensity of these bands varied between different tissues, and the pattern of intensity variation differed between different antigens (figure 8). Similarly, Western blot analysis demonstrated varying content of PSA in samples of different patients, reflecting divergent degree of BPH in different prostates. Detection of calponin demonstrated the presence of smooth muscle in all prostate samples

included in Western blot analysis, while cytokeratins reflected the presence of glandular epithelial cells in most samples (figure 8).

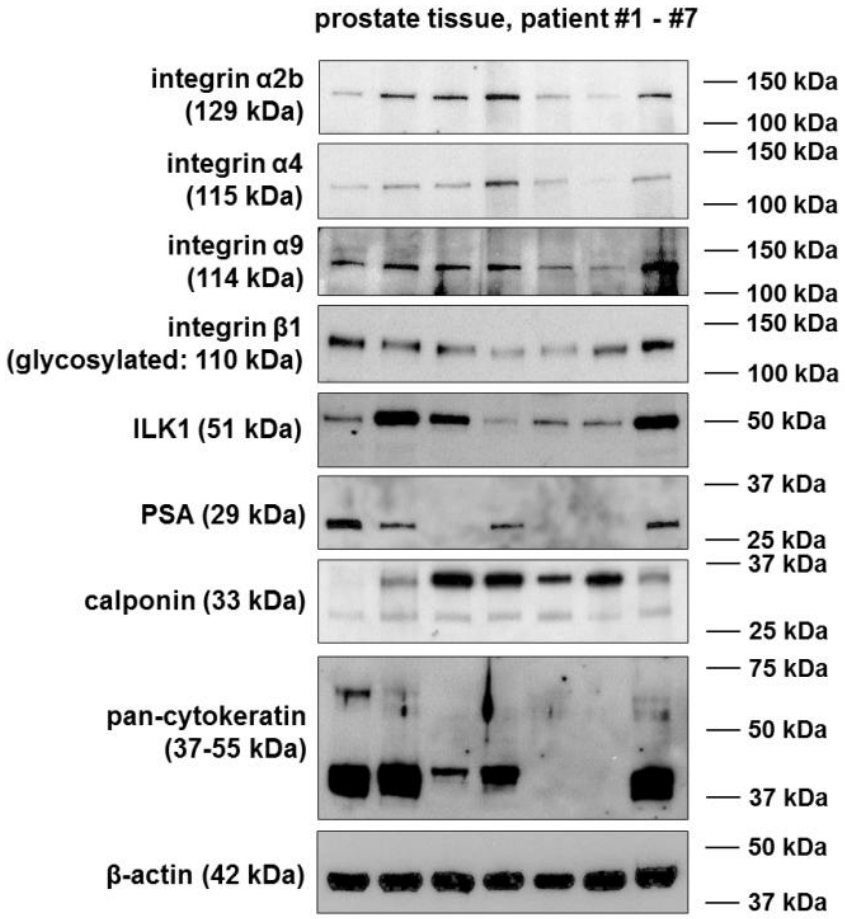

Figure 8: Detection of integrin subunits and ILK in human prostate tissues. Western blot analysis was performed using antibodies for proteins of interest and β-actin serving as housekeeping protein and loading control. The names and sizes of each target protein are noted on the right and positions of marker bands are noted on the right. Shown are blots with all samples being included in Western blot analyses from n=7 patients.

4.2 Detection of the expression and the location of integrins (subunits) of interest and ILK by fluorescence staining

Double stainings of prostate sections were performed using antibodies for integrin α_2, α_4, α_9, β_1 and ILK1, respectively, together with an anti-calponin antibody as a marker of smooth muscle cells, or an anti-pan-cytokeratin antibody as a marker of glandular epithelial cells. Typical prostate histological structures were observed, as the stromal areas showed immunoreactivity to the antibody for calponin, and the surrounding glands showed immunoreactivity to the antibody for pan-cytokeratin in the glandular epithelia. Immunoreactivity for α_2, α_9, β_1 integrin emerged in both the stroma and the glands, where it colocalized with both calponin and pan-cytokeratins (Figure 10, 11, 13), suggesting that integrin subunit α_2, α_9, β_1 are located in smooth muscle, and also in glandular epithelium. Immunoreactivity for ILK1 colocalized with calponin but not pan-cytokeratin (Figure 9), suggesting that ILK1 integrin located in smooth muscle but not in the glandular epithelium. Integrin subunit α_4 emerged in both the stroma and the glandular, but was colocalized with neither calponin nor pan-cytokeratins (figure 12).

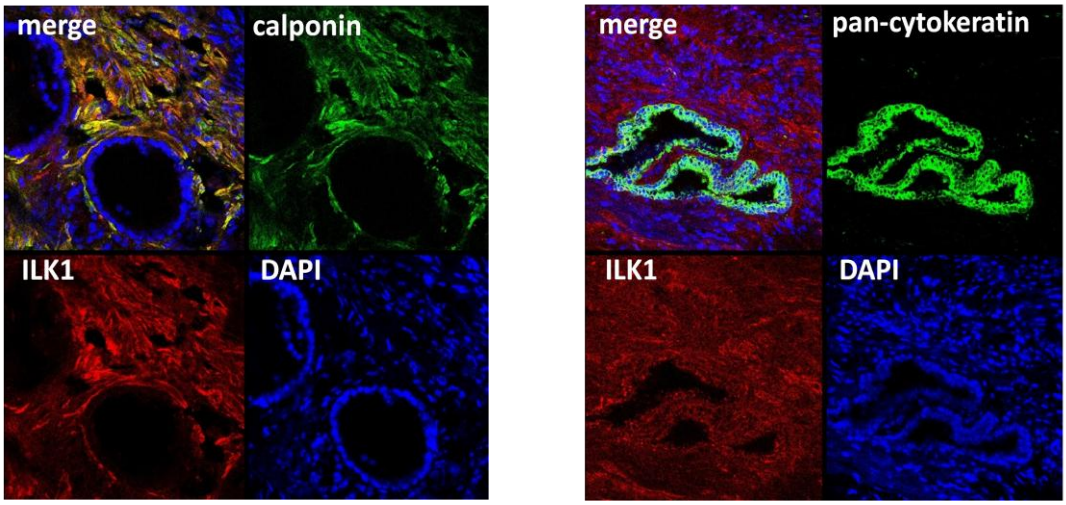

Figure 9: Immunofluorescence staining of human prostate tissues for ILK1. Sections were double-stained with antibodies for ILK1 (indicated as red), in combination with antibodies for calponin (a marker for smooth muscle cells) or pan-cytokeratin (a marker for glandular epithelial cells), both indicated as green. Yellow color in fusion pictures suggests the colocalization of targets. Presented are representative immunofluorescence staining sets from series with tissues from n=5 patients. Negative controls were performed only without primary antibodies (lower panel, right image in each image group).

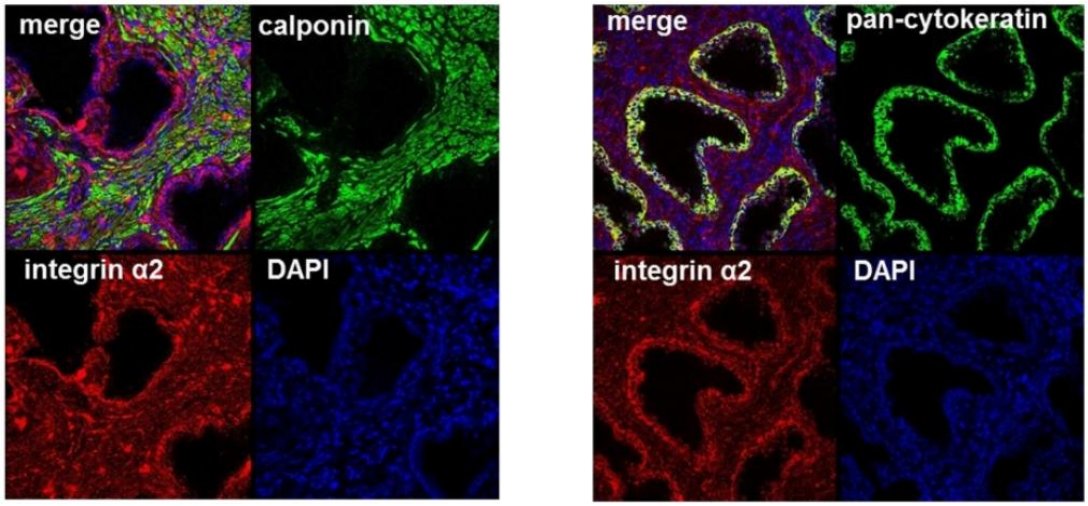

Figure 10: Immunofluorescence staining of human prostate tissues for integrin α_2. Sections were double-stained with antibodies for integrin α_2 (indicated as red), in combination with antibodies for calponin (a marker for smooth muscle cells) or pan-cytokeratin (a marker for glandular epithelial cells), both indicated as green. Yellow color in fusion pictures suggests the colocalization of targets. Presented are representative immunofluorescence staining sets from series with tissues from n=5 patients. Negative controls were performed only without primary antibodies (lower panel, right image in each image group).

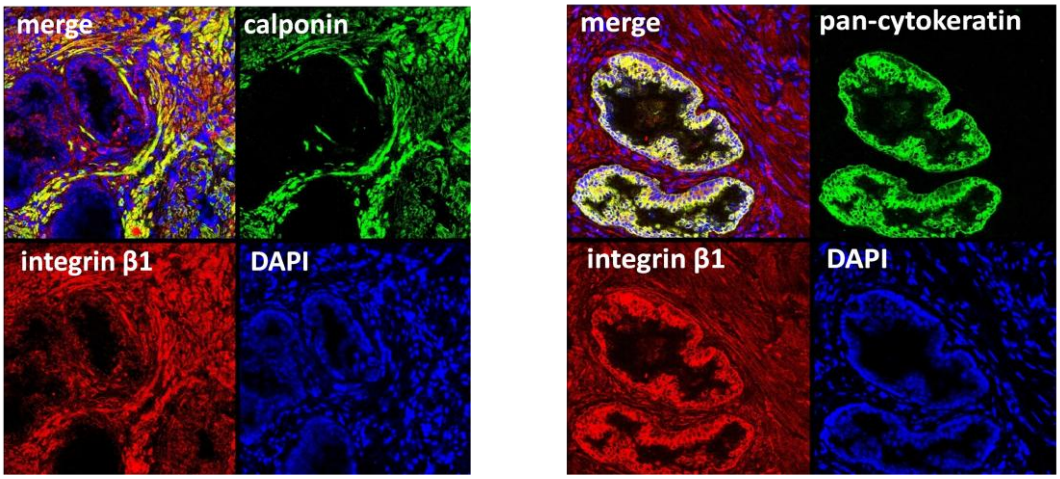

Figure 11: Immunofluorescence staining of human prostate tissues for integrin β_1. Sections were double-stained with antibodies for integrin β_1 (indicated as red), in combination with antibodies for calponin (a marker for smooth muscle cells) or pan-cytokeratin (a marker for glandular epithelial cells), both indicated as green. Yellow color in fusion pictures suggests the colocalization of targets. Presented are representative immunofluorescence staining sets from series with tissues from n=5 patients. Negative controls were performed only without primary antibodies (lower panel, right image in each image group).

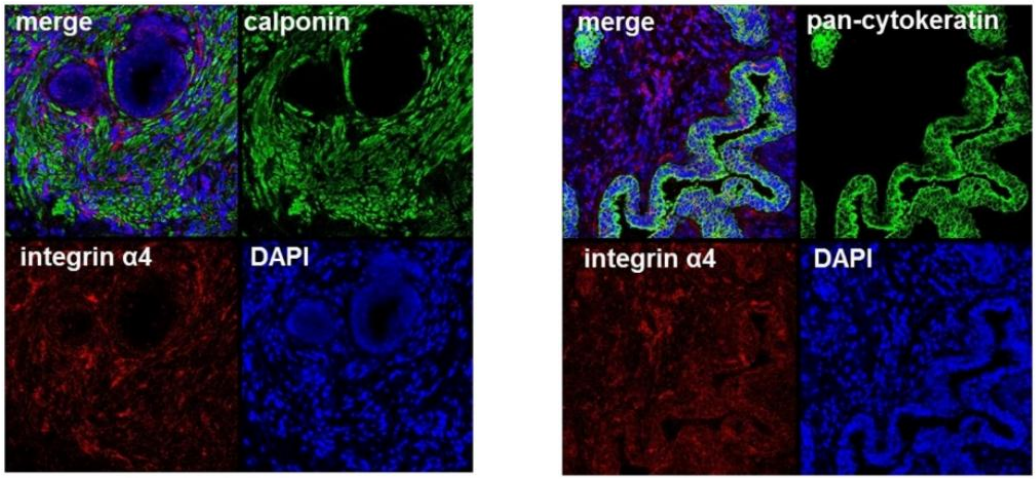

Figure 12: Immunofluorescence staining of human prostate tissues for integrin α_4. Sections were double-stained with antibodies for integrin α_4 (indicated as red), in combination with antibodies for calponin (a marker for smooth muscle cells) or pan-cytokeratin (a marker for glandular epithelial cells), both indicated as green. Yellow color in fusion pictures suggests the colocalization of targets. Presented are representative immunofluorescence staining sets from series with tissues from n=5 patients. Negative controls were performed only without primary antibodies (lower panel, right image in each image group).

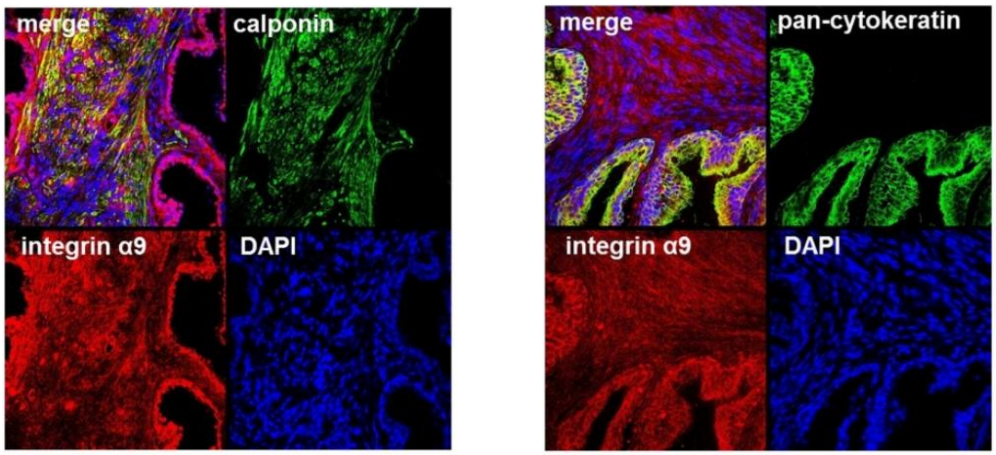

Figure 13 Immunofluorescence staining of human prostate tissues for integrin α_9. Sections were double-stained with antibodies for integrin α_9 (indicated as red), in combination with antibodies for calponin (a marker for smooth muscle cells) or pan-cytokeratin (a marker for glandular epithelial cells), both indicated as green. Yellow color in fusion pictures suggests the colocalization of targets. Presented are representative immunofluorescence staining sets from series with tissues from n=5 patients. Negative controls were performed only without primary antibodies (lower panel, right image in each image group).

4.3 Effect of BTT3033 on prostate smooth muscle contractions.

4.3.1 Effect of BTT3033 on EFS-induced contractions.

EFS (2-32 HZ, 5 frequences proportionally increased) induced contractions in a frequency-dependent way, which were inhibited by BTT3033 (1 μM). Analysis of variance showed significant inhibition by BTT3033 in a whole comparison of two groups ($p<0.01$), and multivariance analysis showed significant inhibition at EFS frequences of 16 HZ ($p<0.05$) and 32 HZ ($p<0.01$)

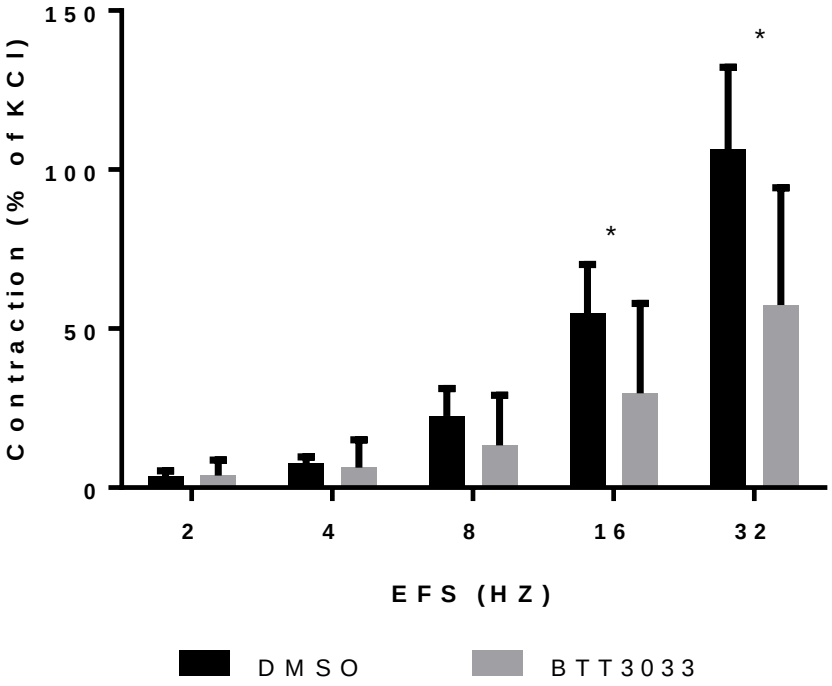

Figure 14. Contractions of human prostate strips measured in the organ bath, which were induced by EFS in proportionally growing frequence. The effects of BTT3033 on EFS-induced contractions were compared with solvent (control) in n=5 independent experiments with tissues from 5 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each frequency of EFS (*$P<0.05$ for control vs. inhibitor of the indicated frequencies).

4.3.2 Effect of BTT3033 on U46619-induced contractions.

U46619 (0.1 to 30 μM, 6 cumulative concentrations) induced contractions in a concentration-dependent way, which were inhibited by BTT3033 (1 μM). Analysis of variance showed significant inhibition by BTT3033 in comparison of whole concentration-response curves ($p<0.03$), while multivariance analysis showed no significant difference at each concentration of U46619.

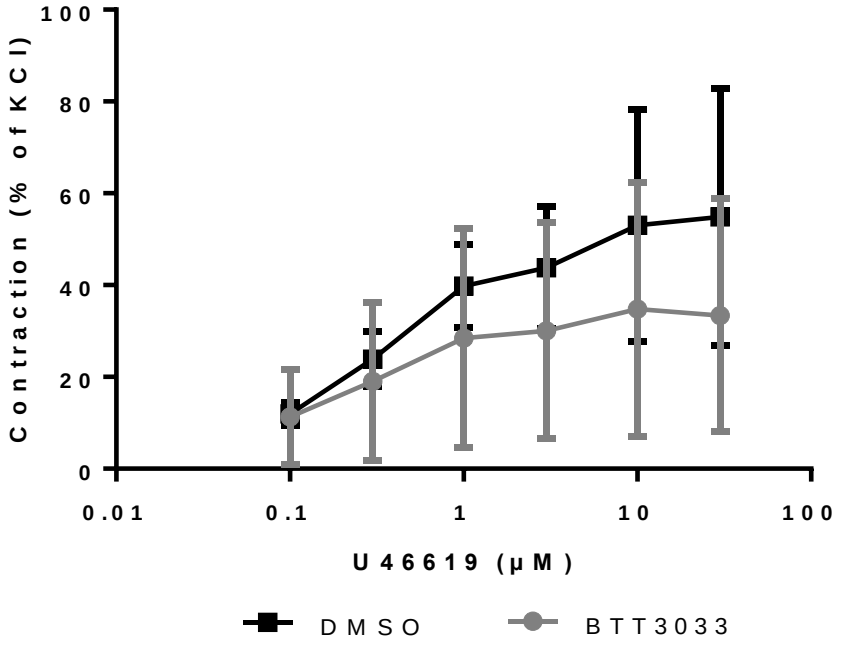

Figure 15. Contractions of human prostate strips measured in the organ bath, which were induced by U46619 in cumulative concentrations. The effects of BTT3033 on U46619-induced contractions were compared with solvent (control) in n=5 independent experiments with tissues from 5 patients. Tissue from each patients was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of U46619.

4.3.3 Effect of BTT3033 on Endothelin-1-induced contractions.

Endothelin-1 (0.1 to 3 μM, 4 cumulative concentrations) induced contractions in a concentration-dependent way, which were not inhibited by BTT3033 (1 μM). Analysis of variance showed no significant inhibition by BTT3033 in comparison of whole concentration-response curves, and multivariance analysis showed no significant inhibition at each concentration of endothelin-1.

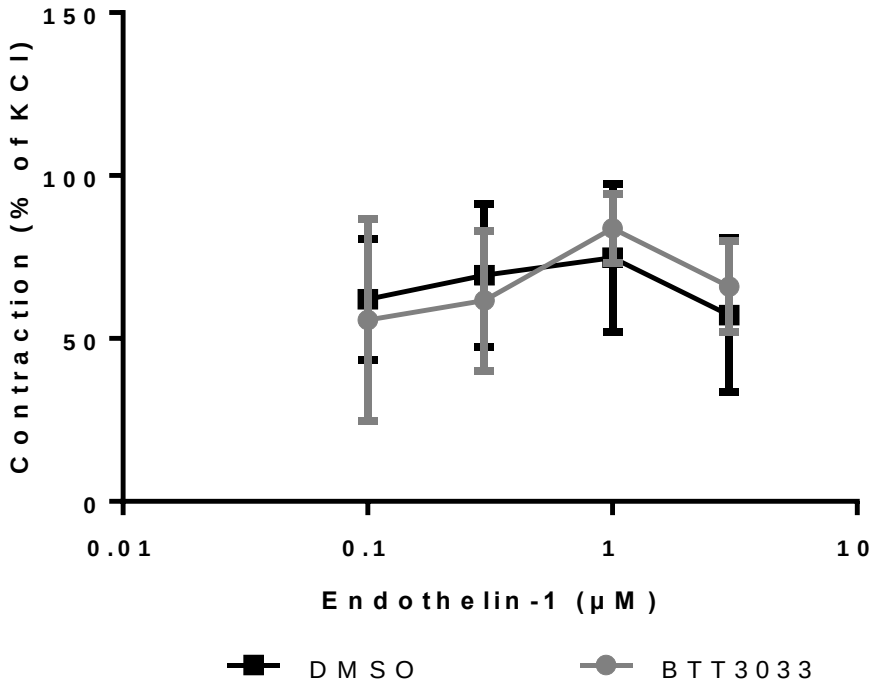

Figure 16. Contractions of human prostate strips measured in the organ bath, which were induced by endothelin-1 in cumulative concentrations. The effects of BTT3033 on endothelin-1-induced contractions were compared with solvent (control) in n=6 independent experiments with tissues from 6 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of endothelin-1.

4.3.4 Effect of BTT3033 on noradrenaline-induced contractions.

Noradrenaline (0.1 to 100 μM, 7 cumulative concentrations) induced contractions in a concentration-dependent way, which were inhibited by BTT3033 (1 μM). Analysis of variance showed significant inhibition by BTT3033 in comparison of whole concentration-response curves ($p<0.01$), and multi-variance analysis showed significant inhibition at noradrenaline concentrations of 3 μM ($p<0.05$) and 10 μM ($p<0.02$).

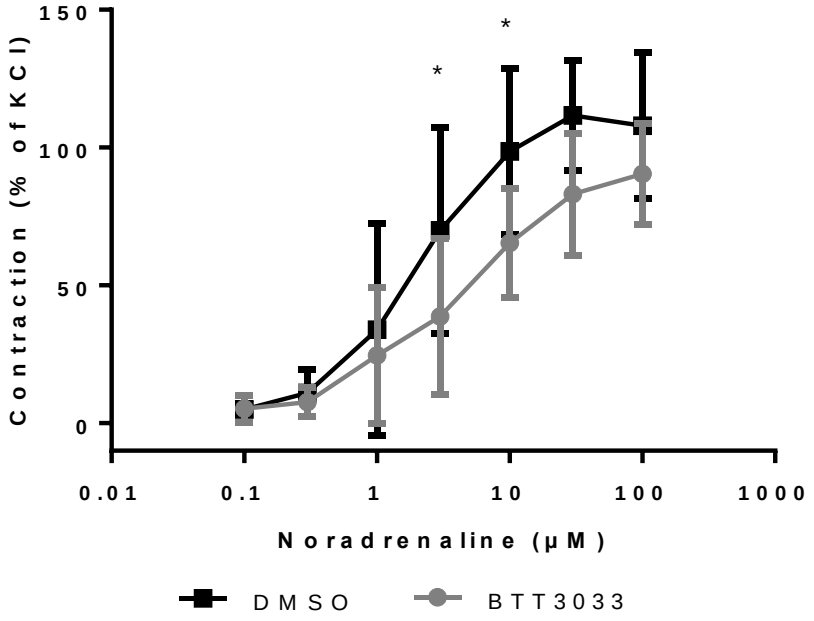

Figure 17. Contractions of human prostate strips measured in the organ bath, which were induced by noradrenaline in cumulative concentrations. The effects of BTT3033 on noradrenaline-induced contractions were compared with solvent (control) in n=5 independent experiments with tissues from 5 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of noradrenaline (*P<0.05 for control vs. inhibitor of the indicated concentrations).

4.3.5 Effect of BTT3033 on phenylephrine-induced contractions.

Phenylephrine (0.1 to 100 μM, 7 cumulative concentrations) induced contractions in a concentration-dependent way, which were not inhibited by BTT3033 (1 μM). Analysis of variance showed no significant inhibition by BTT3033 in comparison of whole concentration-response curves, and multivariance analysis showed no significant inhibition at each concentration of phenylephrine.

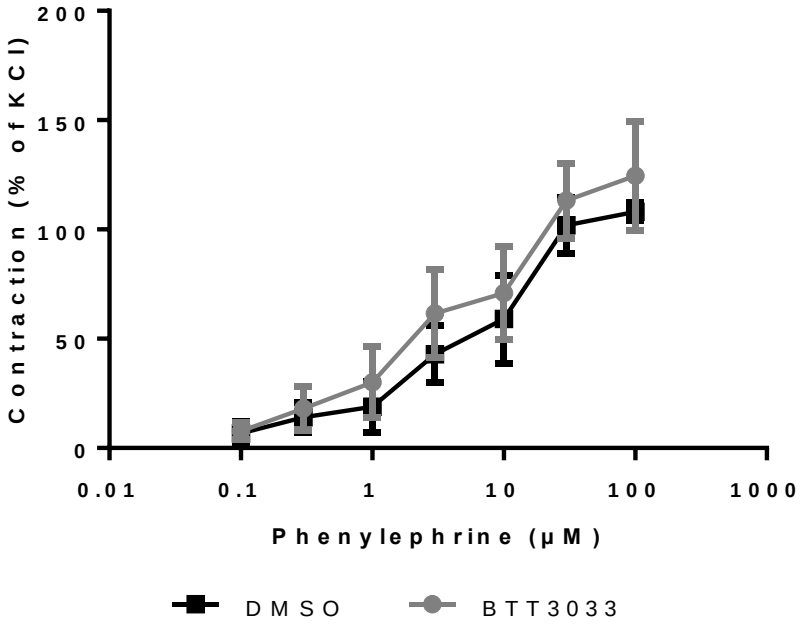

Figure 18. Contractions of human prostate strips measured in the organ bath, which were induced by phenylephrine in cumulative concentrations. The effects of BTT3033 on phenylephrine-induced contractions were compared with solvent (control) in n=6 independent experiments with tissues from 6 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of phenylephrine.

4.3.6 Effect of BTT3033 on methoxamine-induced contractions.

Methoxamine (0.1 to 100 μM, 7 cumulative concentrations) induced contractions in a concentration-dependent way, which were inhibited by BTT3033 (1 μM). Analysis of variance showed significant inhibition by BTT3033 in comparison of whole concentration-response curves ($p<0.03$), while multivariance analysis showed no significant difference at each concentration of methoxamine.

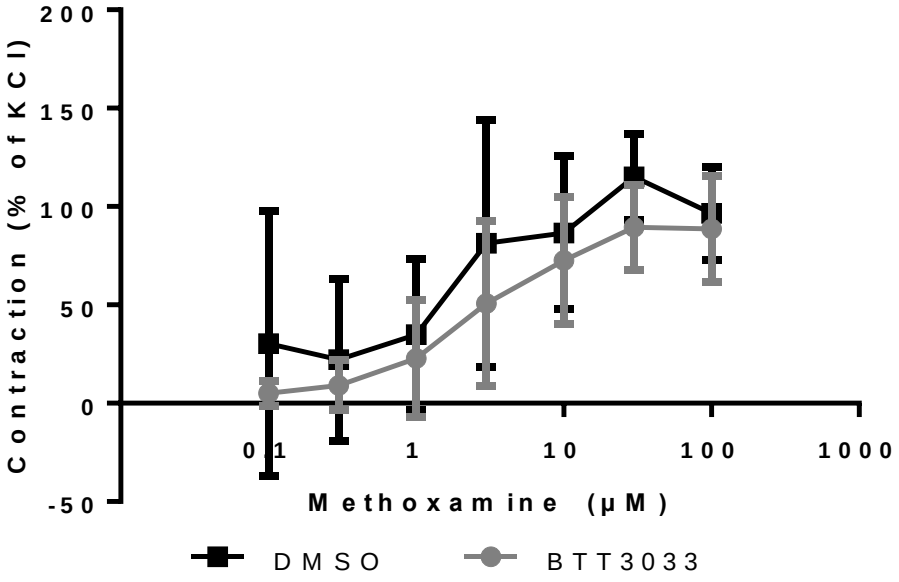

Figure 19. Contractions of human prostate strips measured in the organ bath, which were induced by methoxamine in cumulative concentrations. The effects of BTT3033 on methoxamine-induced contractions were compared with solvent (control) in n=6 independent experiments with tissues from 6 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or

solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of methoxamine.

4.4 Effect of BOP on prostate smooth muscle contractions.

4.4.1 Effect of BOP on EFS-induced contractions.

EFS (2-32 HZ, 5 frequences proportionally increased) induced contractions in a frequency-dependent way, which were not inhibited by BOP (1 μM). Analysis of variance showed no significant inhibition by BOP in comparison of whole concentration-response curves, and multivariance analysis showed no significant inhibition at each frequence of EFS.

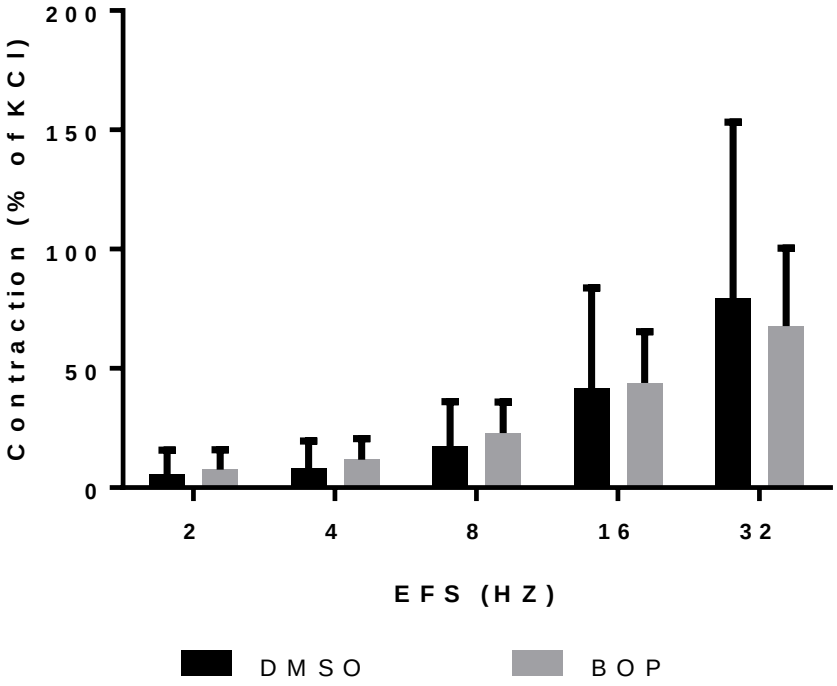

Figure 20. Contractions of human prostate strips measured in the organ bath, which were induced by EFS in proportionally growing frequence. The effects of BOP on EFS-induced contractions were compared with solvent (control) in n=5 independent experiments with tissues from 5 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ±SD on each frequency of EFS.

4.4.2 Effect of BOP on U46619-induced contractions.

U46619 (0.1 to 30 μM, 6 cumulative concentrations) induced contractions in a concentration-dependent way, which were not inhibited by BOP (1 μM). Analysis of variance showed no significant inhibition by BOP in comparison of whole concentration-response curves, and multivariance analysis showed no significant inhibition at each concentration of U46619.

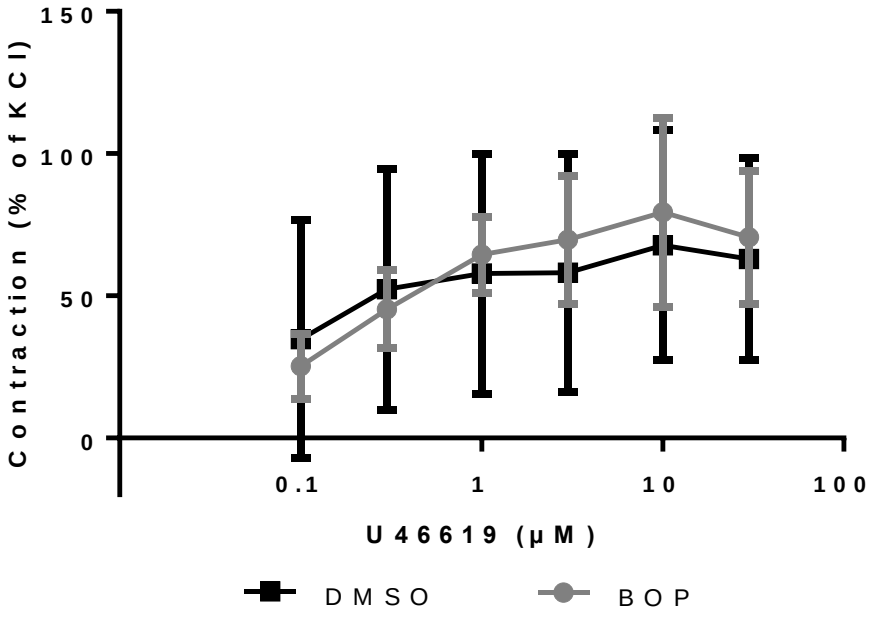

Figure 21. Contractions of human prostate strips measured in the organ bath, which were induced by U46619 in cumulative concentrations. The effects of BOP on U46619-induced contractions were compared with solvent (control) in n=6 independent experiments with tissues from 6 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ±SD on each concentration of U46619.

4.4.3 Effect of BOP on Endothelin-1-induced contractions.

Endothelin-1 (0.1 to 3 μM, 4 cumulative concentrations) induced contractions in a concentration-dependent way, which were not inhibited by BOP (1 μM). Analysis of variance showed no significant inhibition by BOP in comparison of whole concentration-response curves, and multivariance analysis showed no significant inhibition at each concentration of endothelin-1.

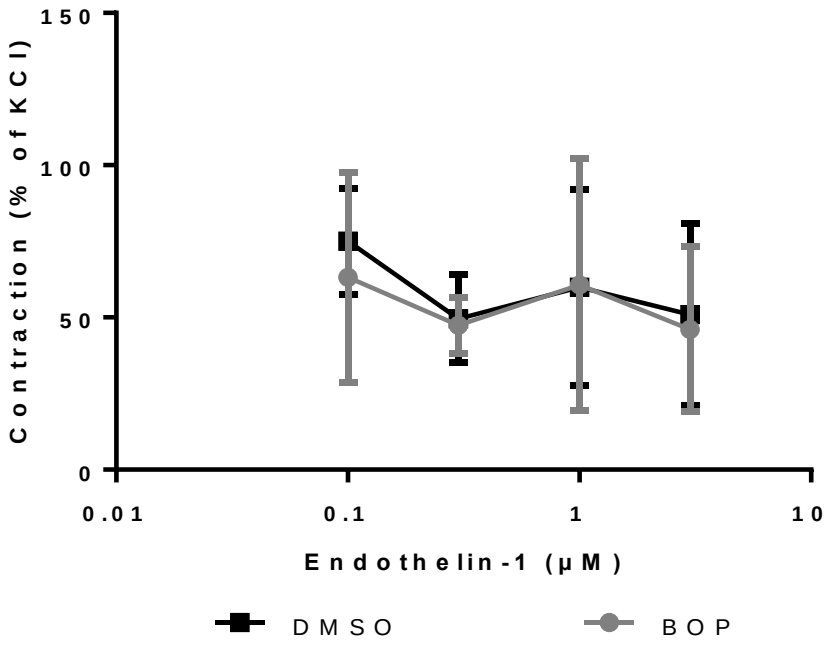

Figure 22. Contractions of human prostate strips measured in the organ bath, which were induced by endothelin-1 in cumulative concentrations. The effects of BOP on endothelin-1-induced contractions were compared with solvent (control) in n=5 independent experiments with tissues from 5 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of endothelin-1.

4.4.4 Effect of BOP on Noradrenaline-induced contractions.

Noradrenaline (0.1 to 100 μM, 7 cumulative concentrations) induced contractions in a concentration-dependent way, which were not inhibited by BOP (1 μM). Analysis of variance showed no significant inhibition by BOP in comparison of whole concentration-response curves, and multivariance analysis showed no significant inhibition at each concentration of noradrenaline.

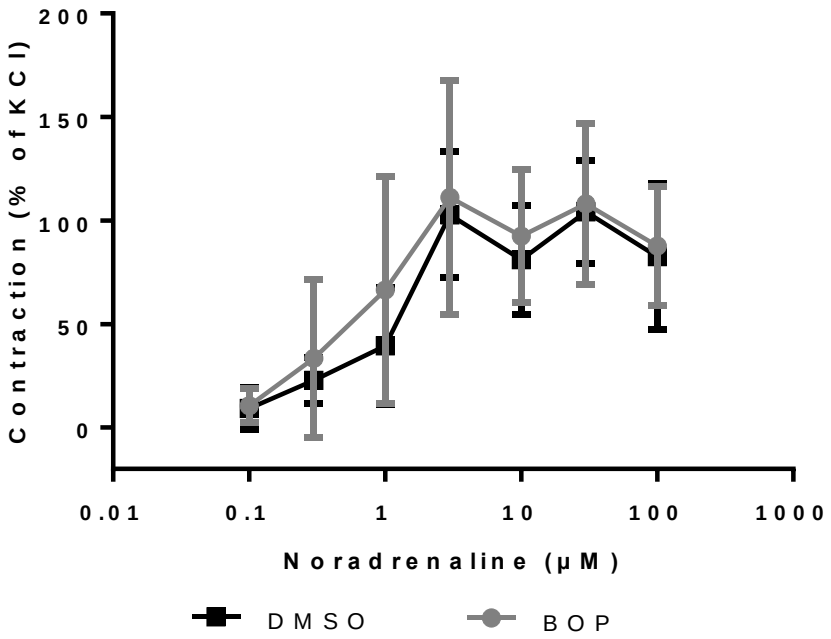

Figure 23. Contractions of human prostate strips measured in the organ bath, which were induced by noradrenaline in cumulative concentrations. The effects of BOP on noradrenaline-induced contractions were compared with solvent (control) in n=6 independent experiments with tissues from 6 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ±SD on each concentration of noradrenaline.

4.4.5 Effect of BOP on phenylephrine-induced contractions.

Phenylephrine (0.1 to 100 μM, 7 cumulative concentrations) induced contractions in a concentration-dependent way, which were not inhibited by BOP (1 μM). Analysis of variance showed no significant inhibition by BOP in comparison of whole concentration-response curves, and multivariance analysis showed no significant inhibition at each concentration of phenylephrine.

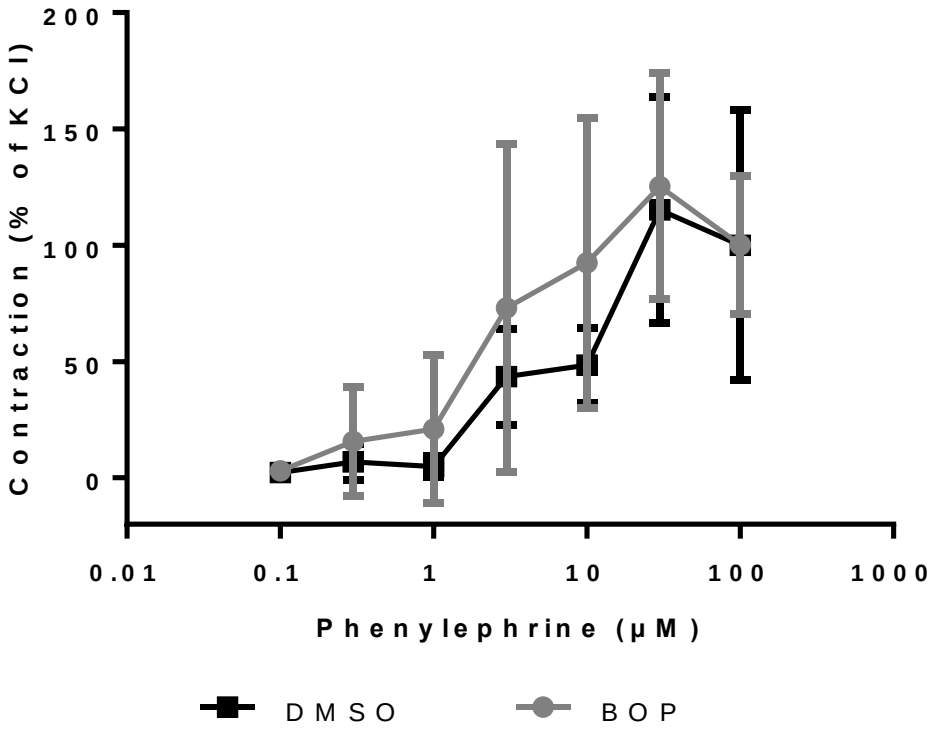

Figure 24. Contractions of human prostate strips measured in the organ bath, which were induced by phenylephrine in cumulative concentrations. The effects of BOP on phenylephrine-induced contractions were compared with solvent (control) in n=6 independent experiments with tissues from 6 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of phenylephrine.

4.4.6 Effect of BOP on methoxamine-induced contractions.

Methoxamine (0.1 to 100 μM, 7 cumulative concentrations) induced contractions in a concentration-dependent way, which were not inhibited by BOP (1 μM). Analysis of variance showed no significant inhibition by BOP in comparison of whole concentration-response curves, and multivariance analysis showed no significant inhibition at each concentration of methoxamine.

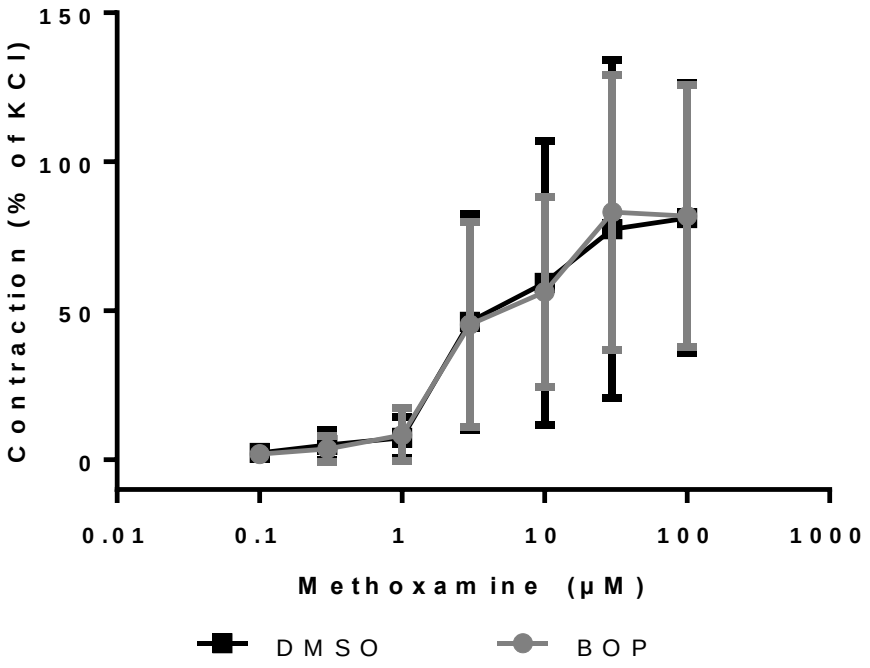

Figure 25. Contractions of human prostate strips measured in the organ bath, which were induced by methoxamine in cumulative concentrations. The effects of BOP on methoxamine-induced contractions were compared with solvent (control) in n=6 independent experiments with tissues from 6 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of methoxamine.

4.5 Effect of Cpd22 on prostate smooth muscle contractions.

4.5.1 Effect of Cpd22 on EFS-induced contractions.

EFS (2-32 HZ, 5 frequences proportionally increased) induced contractions in a frequency-dependent way, which were inhibited by Cpd22 (3 μM). Analysis of variance showed significant inhibition by Cpd22 in a whole comparison of two groups ($p<0.02$), and multivariance analysis showed significant inhibition at EFS frequence of 32 HZ ($p<0.01$)

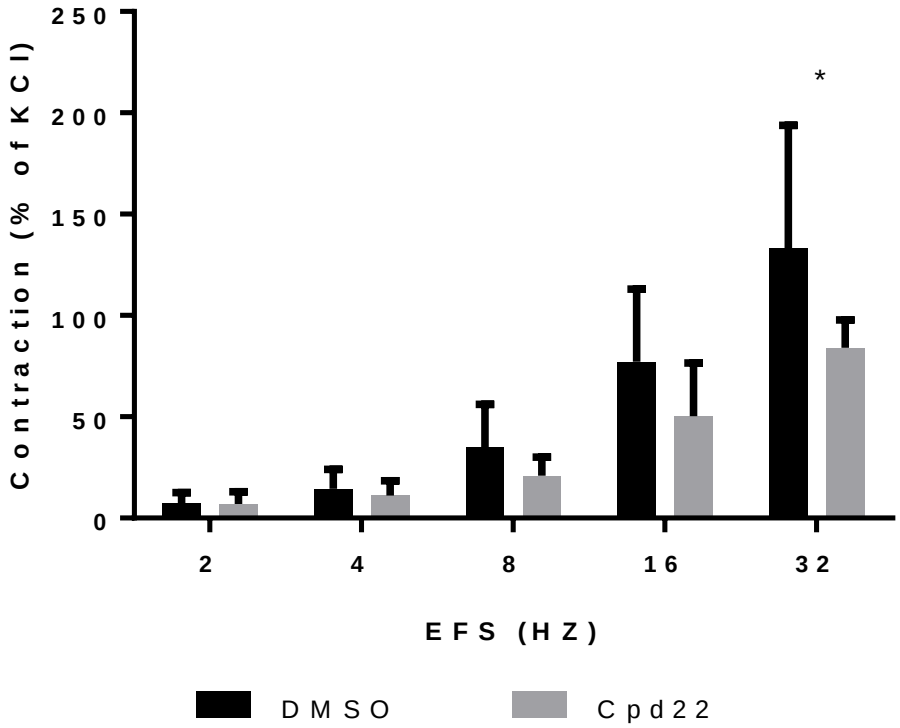

Figure 26. Contractions of human prostate strips measured in the organ bath, which were induced by EFS in proportionally growing frequence. The effects of Cpd22 on EFS-induced contractions were compared with solvent (control) in n=5 independent experiments with tissues from 5 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each frequency of EFS (*P<0.05 for control vs. inhibitor of the indicated frequencies).

4.5.2 Effect of Cpd22 on U46619-induced contractions.

U46619 (0.1 to 30 μM, 6 cumulative concentrations) induced contractions in a concentration-dependent way, which were inhibited by Cpd22 (3 μM). Analysis of variance showed significant inhibition by Cpd22 in comparison of whole concentration-response curves ($p<0.01$), and multivariance analysis showed significant inhibition at U46619 concentration of 3 μM ($p<0.04$) 10 μM ($p<0.02$) 30 μM ($p<0.02$)

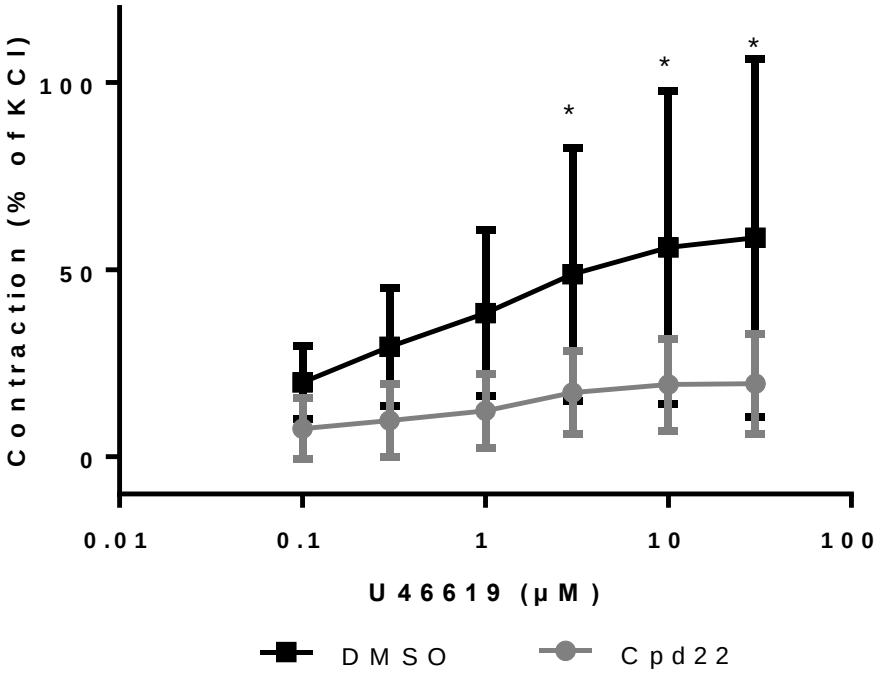

Figure 27. Contractions of human prostate strips measured in the organ bath, which were induced by U46619 in cumulative concentrations. The effects of Cpd22 on U46619-induced contractions were compared with solvent (control) in n=5 independent experiments with tissues from 5 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of U46619 (*$P<0.05$ for control vs. inhibitor of the indicated concentrations).

4.5.3 Effect of Cpd22 on Endothelin-1-induced contractions.

Endothelin-1 (0.1 to 3 μM, 4 cumulative concentrations) induced contractions in a concentration-dependent way, which were not inhibited by Cpd22 (3 μM). Analysis of variance showed no significant inhibition by Cpd22 in comparison of whole concentration-response curves, and multivariance analysis showed no significant inhibition at each concentration of endothelin-1.

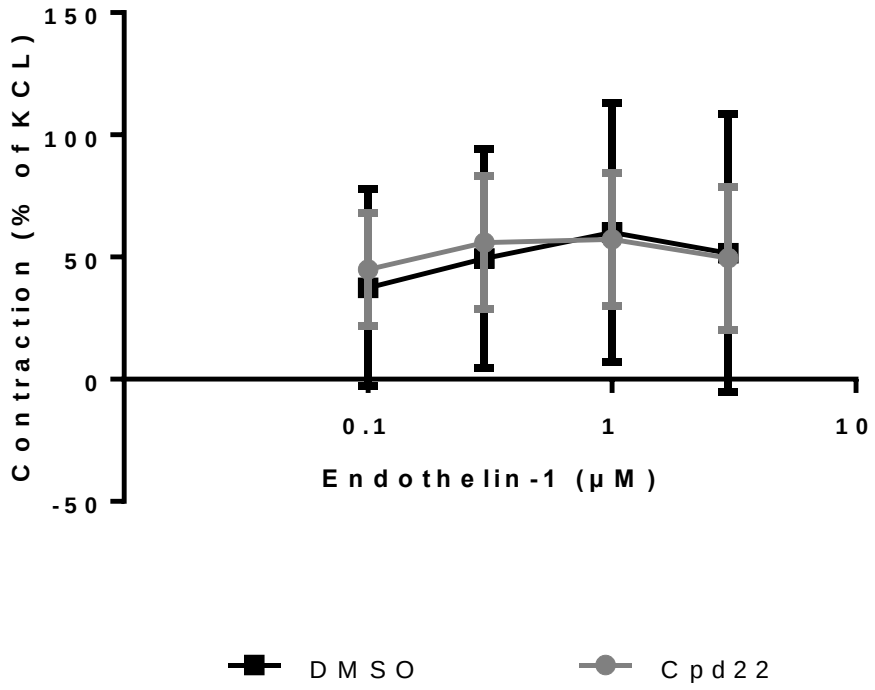

Figure 28. Contractions of human prostate strips measured in the organ bath, which were induced by endothelin-1 in cumulative concentrations. The effects of Cpd22 on endothelin-1-induced contractions were compared with solvent (control) in n=8 independent experiments with tissues from 8 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of endothelin-1.

4.5.4 Effect of Cpd22 on Noradrenaline-induced contractions.

Noradrenaline (0.1 to 100 μM, 7 cumulative concentrations) induced contractions in a concentration-dependent way, which were inhibited by Cpd22 (3 μM). Analysis of variance showed significant inhibition by Cpd22 in comparison of whole concentration-response curves ($p<0.01$), and multivariance analysis showed significant inhibition at noradrenaline concentration of 3 μM ($p<0.05$) and 10 μM ($p<0.02$).

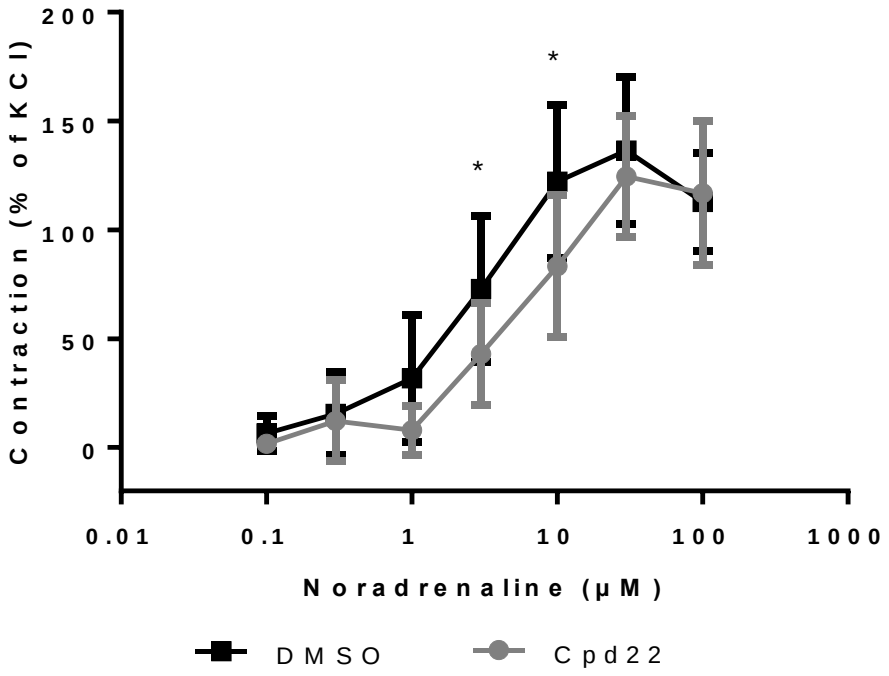

Figure 29. Contractions of human prostate strips measured in the organ bath, which were induced by noradrenaline in cumulative concentrations. The effects of Cpd22 on noradrenaline-induced contractions were compared with solvent (control) in n=6 independent experiments with tissues from 6 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of noradrenaline (*P<0.05 for control vs. inhibitor of the indicated concentrations).

4.5.5 Effect of Cpd22 on phenylephrine-induced contractions.

Phenylephrine (0.1 to 100 μM, 7 cumulative concentrations) induced contractions in a concentration-dependent way, which were not inhibited by Cpd22 (3 μM) phenylephrine. Analysis of variance showed no significant inhibition by Cpd22 in comparison of whole concentration-response curves, and multivariance analysis showed no significant inhibition at each concentration of phenylephrine.

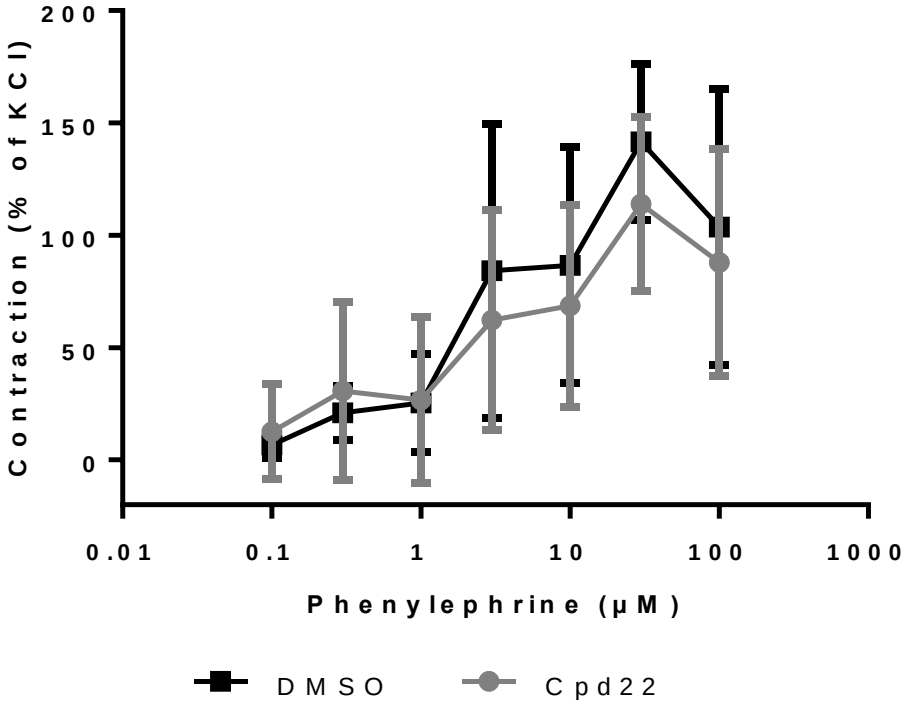

Figure 30. Contractions of human prostate strips measured in the organ bath, which were induced by phenylephrine in cumulative concentrations. The effects of Cpd22 on phenylephrine-induced contractions were compared with solvent (control) in n=6 independent experiments with tissues from 6 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of phenylephrine.

4.5.6 Effect of Cpd22 on methoxamine-induced contractions.

Methoxamine (0.1 to 100 μM, 7 cumulative concentrations) induced contractions in a concentration-dependent way, which were inhibited by Cpd22 (3 μM). Analysis of variance showed significant inhibition by Cpd22 in comparison of whole concentration-response curves ($p<0.02$), and multivariance analysis showed significant inhibition at methoxamine concentration of 100 μM ($p<0.04$).

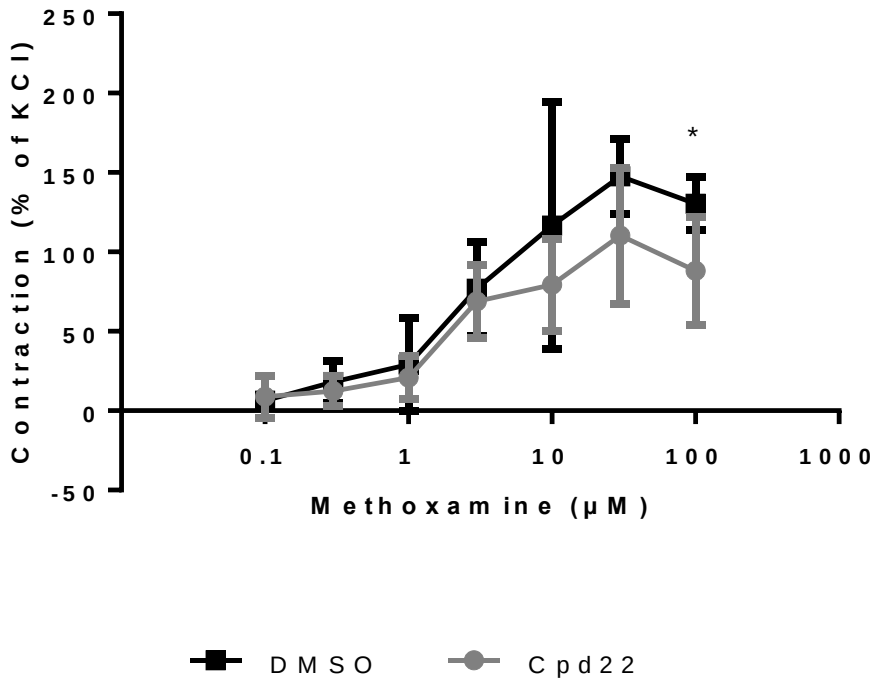

Figure 31. Contractions of human prostate strips measured in the organ bath, which were induced by methoxamine in cumulative concentrations. The effects of Cpd22 on methoxamine-induced contractions were compared with solvent (control) in n=5 independent experiments with tissues from 5 patients. Tissue from each patient was allocated to 4 samples, from which 2 were allocated to the control group, and the 2 others to the inhibitor group. Tissues were incubated with inhibitors (inhibitor group) or solvent (control group) after KCl-induced contractions were measured. Tensions were calculated as % of 80 mM KCl-induced contractions to eliminate the effect of heterogeneities on tissue contractions. Data are presented as means ± SD on each concentration of methoxamine (*P<0.05 for control vs. inhibitor of the indicated concentrations).

5 Discussion

In this thesis, it is demonstrated that the integrin $\alpha_2\beta_1$ inhibitor BTT3033 (1 μM) and the ILK inhibitor Cpd22 (3 μM) can inhibit neurogenic and thromboxane A_2-induced human prostate smooth muscle contraction ex vivo. Very slight inhibitions were observed on noradrenaline- and methoxamine-induced contractions as well, which may be probably neglectable, as the inhibiton extent was limited. According to current literature, this is the first study investigating the effects of integrin and ILK inhibitors on the regulation of smooth muscle contraction in the human prostate.

BTT3033 is a sulfonamide derivative, and a specific inhibitor for $\alpha_2\beta_1$ integrin, binding to the α_2I domain. In an in vitro assay assessing collagen I binding function, the EC_{50} of BTT3033 was 130 nM for $\alpha_2\beta_1$, and 8-fold higher for $\alpha_1\beta_1$, while no inhibition for other integrins was reported [129]. Considering the inhibitor was applied to tissue strips in the organ bath for this thesis, instead to cells in culture wells, 1 μM BTT3033 could be assumed to cause selective inhibition of $\alpha_2\beta_1$, and on a limited extent of $\alpha_1\beta_1$.

BOP is a dual inhibitor for integrins $\alpha_4\beta_1$ and $\alpha_9\beta_1$. Since the IC_{50} for $\alpha_4\beta_1$ and $\alpha_9\beta_1$ is in the subnanomolar range [130], 1 μM BOP was assumed to effectively inhibit both $\alpha_4\beta_1$ and $\alpha_9\beta_1$.

Cpd22 is a highly selective inhibitor for ILK, with an IC_{50} of 0.6 μM. In fact, EC_{50} values in ex vivo tissues may be higher than in cultured cells (or than IC_{50} values in biochemical assays), as the inhibitors have to penetrate interstitial space between cells and extracellular compartments, or have to cross similar barriers [77]. It is assumed that 3 μM Cpd22 mainly caused inhibition of ILK and to limited extent of Abl, CDK1 and p70S6K, so that the inhibition of the prostate smooth muscle was supposed to mainly due to ILK inhibition [131].

A crucial strategy to treat LUTS due to BPH, is to inhibit prostate smooth muscle contraction [100, 111]. Although the principal role of smooth muscle in the prostate is to squeeze the fluid from prostate glands into the urethra, which is mainly triggered by the neurogenic release of noradrenaline, it also compresses the urethra and causes voiding dysfunction in BPH patients. Reducing the basic tone of prostate smooth muscle in BPH can relieve the pressure from the prostate to the urethra.

The basic tone is now believed to be regulated by not only neurogenic and adrenergic signals, but also by non-adrenergic mediators.

Alpha$_1$-blockers reduce neurogenic- and adrenergic-induced contraction. However, up to 69 % of patients are not satisfied by treatment with α_1-blockers and still 36-45 % of patients are not satisfied even using a combination therapy of α_1-blockers with 5-ARIs [22, 23, 77, 78]. On the one hand, in the human body, α_1-blockers will bring a lack of reaction to the sympathetic nerve and possible unsatisfied sensation of ejaculation. On the other hand, in the course of urination, the vagus nerve is the one who dominates instead of the sympathetic nerve, which means neurogenic and adrenergic factors may contribute not so much as assumed in regulating basic tone, which may be the reason why merely targeting α_1 receptor is not enough satisfying even combined with 5 ARIs [76, 79, 132].

Apart from α_1-blockers, various receptors or kinases were shown to be involved in the prostate smooth muscle contraction, in addition to the well established and often characterized receptors and pathways, i. e. α_1-adrenoceptors, calcium, PKC, and RhoA/ROCK. These pathways are at the same time (at least partially) shared by smooth muscle outside the lower urinary tract, what may cause potential side effects in other systems

if they are regarded as targets for male LUTS. Thus, the ideal targets for treating male LUTS should be relatively selective for smooth muscle, and only be located to the lower urinay tract system. The integrin family have a large diversity. Their functions suggest that integrins may have effects in a "cell- and integrin-type specific manner" [133]. Various isoforms of integrins are widely expressed and heterogeneously distributed in different organs. Consequently, organ-specific inhibition may be achieved by inhibitors for certain integrin types. Consequently, organ-specific integrin equipment, organ-specific integrin patterns, and organ-specific integrin inhibition may be achieved by inhibitors for certain integrin types.

Integrins are responsible for cell adhesion, and have been widely investigated in different physiological and pathological processes like immune reaction and thrombosis, and some of them have been put into clinical or preclinical use [121]. For now, $\alpha_{IIb}\beta_3$ integrin in platelets has been proven as a clinically successful drug target for intervention of thrombosis after percutaneous coronary intervention, while medications addressing $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins are reported to treat multiple sclerosis and inflammatory bowel disease [134]. Thus, integrins are in fact regarded and examined as targets with realistic translational value.

Concerning contraction, integrins are essential mechanosensory receptors and β_1-assembling integrins are among the most studied ones [133, 135, 136]. This may offer promising approaches to attain prostate-specific inhibition of smooth muscle contraction, providing that integrins regulate contraction in prostate smooth muscle. Various β_1 integrin heterodimers have been revealed to be involved in smooth muscle contraction in different organs, but not in the prostate yet. Studies showed that topical use of the inhibitor of fibronectin-binding integrin $\alpha_5\beta_1$ facilitated relaxation of the airway, and blockage of laminin-binding integrin $\alpha_7\beta_1$ reduced the contractile phenotype of airway smooth muscle cells [102, 124]. Another study reported that integrin $\alpha_8\beta_1$ mediated the effects of milk fat globule epidermal growth factor-like 8 (Mfge8) on preventing IL-13-induced increases in airway contractility [137, 138]. Liu et al. reported that smooth muscle cell-specific deletion of integrin β_1 reduced the ability of vasoconstriction [139]. Inhibition of $\alpha_5\beta_1$ integrin has also been shown to decrease myogenic constriction using either inhibitors or antibodies [140-142]. It has also been found that knockdown of integrin β_4 in vascular endothelial cells reduced sphingosylphosphorylcholine-induced vascular smooth muscle cells contractions [143]. Thus, the findings presented here for prostate smooth muscle are in line with those previous findings for smooth muscle in other organs.

According to research on expression profiles of more than 90% of all genes in 32 tissues and organs, integrin β_1 is highly expressed in smooth muscle and the fragments per kilobase of transcript per million fragments mapped (FPKM) was 437.32, while the mean FPKM of integrin β_1 among all tested tissue is 145.75 [144]. In that study, the FPKM of integrin β_1 is 255.78 in bladder tissues and 163.44 in prostate tissues [144]. Its expression in the prostate was also suggested by Western blot and immunofluorescence in this study, but the role of integrin β_1 or its integrins in the contraction of smooth muscle in the lower urinary tract system has to the best of my knowledge not been suggested before, and for the first time in this study. Among the β_1 containing integrins, integrin $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_{10}\beta_1$, $\alpha_{11}\beta_1$ can be regarded as receptors for laminin and collagen and can mediate adhesion between cells to ECM [121]. Integrins $\alpha_4\beta_1$and $\alpha_9\beta_1$ can be bound by ligands like VCAM-1, osteopontin, ADAM and mediate adhesion between cells [121, 133]. In this study contraction measurements showed that the integrin $\alpha_2\beta_1$ inhibitor BTT3033 inhibited prostate smooth muscle contraction, mainly on neurogenic and thromboxane-induced contractions, while the integrin $\alpha_4\beta_1$/ $\alpha_9\beta_1$ inhibitor BOP did not inhibit contractions.

Apart from mediating adhesion mechanically, integrins serve to transmit signals outside-in and also inside-out. ILK, as a binding partner for the

integrin β cytoplasmic tail, is one of the kinases that interact with intracellular parts of integrins and process signals from the integrins [145], along with FAK, Src, and other kinases getting evolved in various regulations of cell functions. ILK, on the other hand, can reversely also influence the assembling and function of integrins. ILK is a serine/threonine kinase, which interacts with the intracellular part of integrin β_1 and phosphorylates integrin β_1 [146]. Like integrins, ILK has been associated with various roles in functions like adhesion, proliferation, apoptosis and motility [146] [147].

Integrin-linked kinase controls retinal angiogenesis [148], and mediates healing [149], platelet function [150, 151] and liver regeneration [152, 153]. It was revealed that loss of ILK from mouse liver cells brought about apoptosis and hepatitis [154]. In the tumor research area, ILK turned out to be crucial in cancer formation and progression [146, 155]. ILK protein levels are increased in several human cancers and often the expression level predicts poor patient outcome [156]. ILK was found to be activated in acute myeloid leukemia [157], and it has been found that in multiple myeloma ILK is also a potential target [158].

ILK is required for polarizing the epiblast, cell adhesion, and controlling

actin accumulation [159]. Moreover, ILK was found to play roles in cardiomyocytes and in cardiac smooth muscle [156]. Studies regarding the heart showed that ILK mediates development and contraction of the heart [82] and gets involved in cardiac hypertrophy, pulmonary hypertension or other diseases [160, 161]. Studies also found that ILK regulates smooth muscle contraction in gut and may be involved in pulmonary hypertension [162]. However, to the best of my knowledge, ILK has not been investigated in smooth muscle contraction of the prostate yet either. Here, the ILK inhibitor Cpd22 showed inhibition effects on prostate smooth muscle contraction, mainly on neurogenic and thromboxane-induced contractions.

The results of Western blot and immunofluorescence in this thesis suggest that ILK1 and integrin subunits α_2 and β_1 are expressed in the prostate stroma as they colocalize with the smooth muscle marker caponin. In addition, α_4 and α_9 was also expressed in the prostate stroma. However, the dual integrin $\alpha_4\beta_1$/ $\alpha_9\beta_1$ inhibitor BOP had no effect on contraction in this study. Consequently, results suggesting expression must not necessarily guarantee a function in the field of contraction. Testing the ex vivo effects of inhibitors in the organ bath can provide direct evidence of contraction inhibition.

The functions of integrins and ILK profoundly rely on cell-cell and cell-ECM interactions in the presence of Ca^{2+}, Mg^{2+} or Mn^{2+} [163] [164-166], which can not be mimicked precisely in cell culture. Although efforts have been made through adjusting ion concentration and alteration to 3D culture, contraction studies in organ bath are still more close to the natural condition, with the results more intuitive and direct. Thus, although cell culture studies can also indicate contractility using contraction assays, findings and conditions may be less physiological than those using ex vivo tissues or in vivo studies.

Human prostate tissues from radical prostatectomy used here were taken from the periurethral zone, where tumors are rare. Although the most ideal tissue for studies like this would be from BPH patients, surgeries handling BPH (i.e. ablative approaches) destroy and heat the tissue. Thus, tissues would be traumatized and can not serve to be investigated by contraction assay and molecular studies anymore. Animal prostate tissues are commonly used in organ baths for contraction assay. However, the findings in animal models can not always be reproduced in studies using human tissues. Taking detrusor as examples, studies showed that ATP-purinergic contraction in an important part of sympathetic nerve mediated contraction, however, there has never been a study indicating ATP or its analogues can induce contraction in human bladder tissue or

prostate [167].

Human prostate tissues show high heterogeneity among individuals. This can be seen from the wide error bars of contraction assays using human prostate compared with those using animal model. In this study, the high individual variation is also well reflected by Western blot analyses showing very high heterogeneity of calponin and pan-cytokeratin expression levels, which refers to the heterogeneity of smooth muscle and glandular composition, and diverging degree of BPH (as shown by the varying content of PSA). Although this may also result from sampling, experimental design should avoid the influence of heterogeneity. Consequently, four pieces of prostate tissue from the same patient were allocated to 2 groups in each set of experiment to avoid individual differences. Secondly, contractions were expressed as % of high molar KCl-induced contraction to avoid heterogeneity resulting from individual variation from sampling, or from different content and condition of smooth muscle, or from different sample sizes.

BTT3033 and Cpd22 showed clear inhibition of neurogenic, and thromboxane A_2-induced contractions. Only very slight inhibitions were observed on noradrenaline- and methoxamine-induced contractions as

well, which may be neglectable, as the extent of any inhibition was very small and data for different α_1-adrenergic agonists were partially inconclusive. Interestingly, BTT3033 and Cpd22 showed the same pattern of contraction inhibition. Instead of considering those differences as coincidence or randomity, a better explanation is that integrins /ILK may regulate prostate smooth muscle in concert, and different from the current mainstream signaling pathway.

BTT3033 and Cpd22 showed different results on U46619- and endothelin-1-induced contractions, although these are both non-adrenergic agonists. The reason may be differences at the two different receptors they bind. U46619 and endothelin-1 activate the thromboxane A2 receptor and endothelin receptors, respectively. Both are G protein-coupled receptors, however, the former has been supposed to couple predominately with $G\alpha_{12/13}$, and endothelin-receptors predominately with $G\alpha_{q/11}$ [168, 169]. According to current studies, $G\alpha_{q/11}$ activates phospholipase Cβ (PLCβ), and mediates contraction mostly via Ca^{2+}- and PCK-dependent pathway, while $G\alpha_{12/13}$ may further activate the guanine nucleotide exchange factor p115 RhoGEF (GEF) and mediates contraction in a Ca^{2+}-independent, but RhoA/Rock-dependent pathway [169, 170]. Notably, it has been reported that ILK is responsible for Ca^{2+} independent vascular smooth muscle contraction through directly diphosphorylation of myosin, which was

insensitive to the MLCK inhibitor AV25 [171, 172]. According to the theory of the publications above, endothelin-1 mainly induces Ca^{2+}-dependent MLCK activation, and is relatively resistant to ILK inhibition, considering MLCK/MLCP activity ratio is crucial. However, U46619 mainly induces contraction via Ca^{2+} independent MLCP deactivation, so that the ILK/MLCP activity ratio may be a more crucial factor and sensible to Cpd22. However, not many studies supported or tried to disprove it. A study performed with human prostate smooth muscle showed that U46619 may induce prostate smooth muscle contraction via both Ca^{2+}-dependent and -independent pathways [109]. This study is more in line with the formers, and may further indicate the possibility that integrins like integrin $\alpha_2\beta_1$ mediate smooth muscle contraction via ILK.

Noradrenaline, methoxamine, and phenylephrine activate the same receptor, namely α_1-adrenoceptors, so that the divergent effects of BTT3033 and Cpd22 on noradrenaline- and methoxamine-induced contractions on the one hand, and phenylephrine-induced contractions on the other hand, is harder to explain, although our findings are conclusive to that effect, that any effect of BTT-3033 and Cpd22 on α_1-adrenergic contractions is very small, if occuring at all. However, divergent pharmacological profiles of these α_1-adrenergic agonists may be still worth discussing. One of the clues is that in some studies concerning

neurotransmitter release on rat superior cervical ganglion cells, phenylephrine but not methoxamine will increase fractional noradrenaline efflux, which can not be blocked by α_1-adrenergic receptor antagonists or by removal of extracellular calcium [20, 173]. Besides, methoxamine can bind to α_{1b}-adrenoceptors, while noradrenaline and phenylephrine can not [60]. Certainly, two divergent results for phenylephrine and other α_1-adrenergic agonists show that the mechanism of regulation of smooth muscle contraction are much more complicated than currently illustrated and needs to be further revealed [76].

Alpha$_1$-blockers only inhibit neurogenic and α_1-adrenergic contractions, but not others. New options are expected to target also non-adrenergic contractions. The effect of PDE inhibitors cover the non-adrenergic contractions, but provide efficacy that is not satisfying enough [80]. The inhibitors in this study were able to reduce not only neurogenic but also non-adrenergic thromboxane A2-induced contractions, which have a different spectrum of contraction inhibition than the current α_1-blockers.

Some of the integrins that have not been investigated in this and current studies may also be involved in prostate smooth muscle contraction. Considering the integrin family has a great diversity of distribution and

function, the special integrin barcode may be found with a specific role in prostate smooth muscle contraction, or with limited side effects outside the lower urinary tract from their inhibitors. More studies may be needed to explore various integrins and their effect and specificity in the prostate smooth muscle contraction.

Taken together, this study revealed possible roles of integrin $\alpha_2\beta_1$ and ILK in prostate smooth muscle contraction. The inhibitors for them, i.e. BTT3033 and Cpd22 reduce contractions simulated by EFS, as well as by the non-adrenergic agonist U46619. Their effects of contraction induced by the adrenergic receptor agonists noradrenaline and methoxamine are weak, although significant. They provide a different spectrum of contraction inhibition than α_1-blockers. The consistency of the inhibition pattern between BTT3033 and Cpd22 suggests the possibility that integrin $\alpha_2\beta_1$ mediates contraction via ILK. Integrins and ILK may serve as novel target for treating LUTS suggestive of BPH in vivo. Further studies are expected to investigate the roles of other integrins in the prostate smooth muscle contractions and their specificities, as well as the underlying mechanisms.

Non-adrenergic mediators including thromboxane A2 are suspected to maintain prostate smooth muscle tone and urethral obstruction during

treatment with α1-blockers, what may account for the limited efficacy of α1-blockers. Compounds inhibiting prostate smooth muscle contraction in vitro may be principally considered as promising candidates for in vivo studies, as it may be expected that the inhibition of smooth muscle contraction translates to improvements of LUTS. However, the inhibition of adrenergic contractions observed in our study was small and is probably neglectable in vivo, in contrast to the clear inhibitions of EFS- and U46619-induced contractions. Certainly, the contribution of integrins and ILK to urodynamic regulation can not be estimated until in vivo studies are performed. However, various cardiovascular side effects could limit the use of BTT-3033 or Cpd22 for LUTS treatment. $Alpha_2beta_1$ integrin occurs at different sites in the cardiovascular system. Apart from endothelial and vasuclar smooth muscle cells, it forms a major collagen binding receptor of platelets, also referred to as glycoprotein Ia/IIa, which accounts for platelet adhesion to injured sites at the vessel wall [174, 175]. Consequently, α2β1 critically regulates hemostasis, and promotes thrombus formation and increases the risk of myocardial infarction and stroke [176]. On the other hand, its underexpression is associated with only mildly increased bleeding times but not with severe bleeding, and allows quite normal hemostasis [176]. Accordingly, small molecule α2β1 inhibitors, which were tested as antithrombotic intervention in animal models, may increase bleeding times not more than aspirin [177].

Hemodynamic effects may be rather expected from ILK inhibitors, than from integrin α2β1 inhibition. Thus, in vivo administration of Cpd22 decreased the heart rate and pulmonary vascular resistance [161]. Although no effect on mean arterial pressure was reported, hypotensive side effects still need to be examined, as effects on peripheral or systemic vascular resistance have to the best of our knowledge not yet been addressed [161].

6 Conclusion

Small molecule inhibitors for integrins $\alpha_2\beta_1$ and ILK cause inhibition of neurogenic and thromboxane A2-induced smooth muscle contractions in the human prostate. Inhibition of integrins and ILK to target prostate smooth muscle contraction for treatment of LUTS suggestive of BPH may be possible.

7 Summary

Male LUTS cause heavy burdens, and are commonly attributed to BPH. Prostate smooth muscle tone increases in BPH and often accounts for bladder outlet obstruction, causing the symptoms. Reducing the prostate smooth muscle tone is a crucial strategy for medical LUTS treatment. In the current medical therapy, α_1-blockers are the first option, considering the crucial role of α_1-adrenoceptors in mediating prostate smooth muscle contraction. However, non-adrenergic contraction of prostate smooth muscle is not within the α_1-blockers' spectrum and probably accounts for their limited efficacy.

Integrins are responsible for cell adhesion, anchoring the cell skeleton to other cells or to extra-cellular matrix, what is crucial for smooth muscle contraction. Integrins mediate cell functions through partners like ILK. Integrins and ILK have been shown to be involved in smooth muscle contraction in different body systems. However, according to current available literature, they have never been investigated in prostate smooth muscle contraction by functional experiments.

The integrin $\alpha_2\beta_1$ inhibitor BTT3033, the integrin $\alpha_4\beta_1/\alpha_9\beta_1$ inhibitor BOP and the ILK inhibitor Cpd22 were investigated in the present study. Western blot and immunofluorescence suggested that all related integrin subunits may be expressed in the human prostate, and that they are located to stromal smooth cells. Contraction experiments showed that BTT3033 and Cpd22, but not the integrin $\alpha_4\beta_1/\alpha_9\beta_1$ inhibitor BOP inhibit contractions of human prostate tissues. BTT3033 and Cpd22 shared the same pattern, as they both inhibited prostate smooth muscle contractions induced by the EFS-stimulated neurotransmission and non-adrenergic agonist U46619, with no or very limited inhibition effects on contractions induced by the α_1-adrenergic agonists noradrenaline, methoxamine, and phenylephrine, as well as non-adrenergic agonist endothelin-1.

To the best of my knowledge, this is the first study adressing the effects of integrin and ILK inhibitors on prostate smooth muscle contraction, and suggesting a role of integrins and ILK in human prostate smooth muscle contraction. As integrins show organ-specific composition, integrin inhibitors targeting a prostate-specific integrin code may offer a promising alternative or supplementary approach to attain prostate specific inhibition, without side effects outside the lower urinary tract system.

8 Abbreviations

α_1-blockers	α_1-adrenoceptor antagonists
β_3-agonists	β_3-adrenoceptor agonists
5-ARIs	5α-reductase inhibitors
AC	Adenylyl cyclases
ANOVA	One-way analysis of variance
Arf	ADP ribosylation factor
ATP	Adenosine 5’-triphosphate
AUR	Acute urinary retention
BOO	Bladder outlet obstruction
BPE	Benign prostatic enlargement
BPH	Benign prostatic hyperplasia
BPO	Benign prostatic obstruction
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
CaM	Calmodulin
cAMP	Adenosine 3':5'-cyclic monophosphate
CB1	Cannabinoid receptor type 1

Cdc42	Cell division cycle 42
cGMP	Guanosine 3', 5'-cyclic monophosphate
COX	Cyclooxygenase
CUR	Chronic urinary retention
DAG	Diacylglycerol
DAPI	4’, 6’-diamidino-2-phenylindole-dihydrochloride
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
EAU	European Association of Urology
EC50	Half maximal effective concentration
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EFS	Electric field stimulation
EPAC	Exchange proteins directly activated by cAMP
FAK	Focal adhesion kinase
FRKM	Fragments per kilobase of exon model per million reads mapped
GEFs	Guanine nucleotide exchange factors
GTPase	Guanosine triphosphatase

IC50	Half maximal inhibitory concentration
ILK	Integrin-linked kinase
IP3	Inositol 1,4,5-trisphosphate
IPSS	International Prostate Symptom Score
KCl	Potassium chloride
K-H	Krebs- Henseleit
KH2PO4	Potassium hydrogen phosphate
LUTS	Lower urinary tract symptoms
MAPK	Mitogen-activated protein kinase
Mfge8	Milk fat globule epidermal growth factor-like 8
MgCl2	Magnesium chloride
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
MAPKs	Mitogen-activated protein kinase
MLC	Myosin light chain
MLCK	MLC kinase
MVD	Microvessel density
NaCl	Sodium chloride
NaHCO3	Sodium bicarbonate
NEM	N-Ethylmaleimide

NPY	Neuropeptide Y
OCT	Optimal cutting temperature
PAK	P21-Activated Kinase
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with 0.1% Tween 20
PDE	Phosphodiesterase
PGs	prostaglandins
PKA	Protein kinase A
PKC	Protein kinase C
PLCβ	Phospholipase Cβ
PKG	Protein kinase G
PLK	Polo-like kinase
PPADS	Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate
PSA	Prostate-specific antigen
PTK	Protein tyrosine kinases
Q_{max}	The maximum flow rate
QOL	Quality of life
Rac	Ras-related C3 botulinum toxin substrate
Rap1	Ras-related protein 1

Ran	Ras-related Nuclear protein
Rho	Rhodopsin
RhoGEF	Rho guanine nucleotide exchange factor
SD	Standard deviation
TXA2	Thromboxane A2 (TXA2)
UR	Urinary retention
UTI	Urinary tract infection

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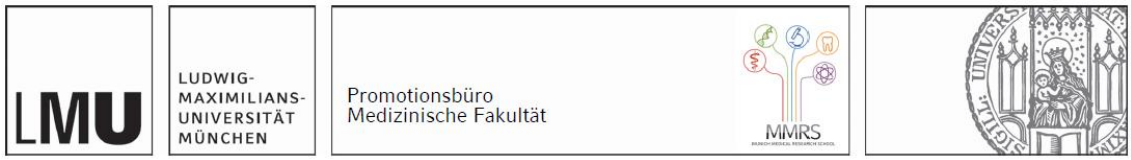

Affidavit

Li, Bingsheng

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Surname, first name

I hereby declare, that the submitted thesis entitled:

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