ORIGINAL ARTICLE

The STK16 inhibitor STK16-IN-1 inhibits non-adrenergic and non-neurogenic smooth muscle contractions in the human prostate and the human male detrusor

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Abstract

Mixed lower urinary tract symptoms (LUTS) (voiding symptoms suggestive of benign prostatic hyperplasia plus storage symptoms, which can be caused by overactive bladder) are common in men. Unwanted contraction of prostate and/or bladder smooth muscle has been implied in the pathophysiology of male LUTS. Here, we examined effects of the serine/threonine kinase 16 (STK16) inhibitor STK16-IN-1 on contraction of human tissues from the prostate and male detrusor. Tissues were obtained from radical prostatectomy and radical cystectomy. Contractions were studied in an organ bath and STK16 expressions by Western blot analyses and fluorescence staining. In prostate tissues, STK16-IN-1 (1 μM) inhibited contractions induced by endothelin-1 and the thromboxane A_2 analog U46619. Contractions of prostate tissues induced by noradrenaline, the α_1-agonists phenylephrine and methoxamine, or electric field stimulation (EFS) were not changed by STK16-IN-1. In male detrusor tissues, STK16-IN-1 inhibited contractions induced by the cholinergic agonists carbachol and metacholine, and contractions induced by U46619. EFS-induced contractions of detrusor tissues were not changed by STK16-IN-1. Western blot analyses of prostate and detrusor tissues revealed bands matching the molecular weight of STK16. Fluorescence staining of prostate tissues using STK16 antibodies resulted in immunoreactivity in smooth muscle cells. STK16-IN-1 selectively inhibits non-adrenergic/non-neurogenic smooth muscle contractions in the male prostate and to limited extent in the bladder. Because non-adrenergic contractions in the male LUTS may account for limited efficacy of α_1-blockers and for α_1-blocker-resistant symptoms, studies assessing add-on of STK16-IN-1 to α_1-blockers in mixed LUTS appear feasible.

Keywords Lower urinary tract symptoms (LUTS) · Benign prostatic hyperplasia (BPH) · Overactive bladder (OAB) · Mixed LUTS · Smooth muscle contraction · STK16-IN-1

Introduction

Male lower urinary tract symptoms (LUTS) include obstructive symptoms suggestive of benign prostatic hyperplasia (BPH) and storage symptoms, which can be caused by an overactive bladder (OAB) (Irwin et al. 2011). While male LUTS were solely attributed to BPH for decades, it is now clear that storage symptoms are common in men, and that many patients show both symptoms at once, referred to as mixed LUTS (Fullhase et al. 2013; Oelke et al. 2013). Obstructive symptoms are often caused by BPH, as increased prostate smooth muscle tone and prostate enlargement in BPH may cause urethral compression, which may result in impaired bladder emptying and symptoms (Hennenberg et al. 2014). Storage symptoms can be caused by involuntary, exaggerated smooth muscle contractions in the urinary bladder wall (detrusor), which may cause urgency and finally incontinence (Andersson and Arner 2004). According to the assumed role of smooth muscle contractions for both symptom complexes, medical treatments aiming to improve LUTS may reduce symptoms by smooth muscle relaxation in the prostate or in the bladder (Andersson 2011; Andersson and Arner 2004;

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Hennenberg et al. 2014; Nambiar et al. 2018; Oelke et al. 2013). The gold standards are α_1-adrenoceptor antagonists (α_1-blockers) for LUTS suggestive of BPH, and muscarinic receptor antagonists (anticholinergics) for OAB, as contractions are induced by activation of α_1-adrenoceptors in the prostate and of muscarinic receptors in the detrusor (Nambiar et al. 2018; Oelke et al. 2013).

Adequate treatment of mixed LUTS and even of the single-symptom complexes still represents a challenge, due to limited efficacies of medications in many patients and unbalanced side effects. On average, α_1-blockers may improve LUTS suggestive of BPH and urinary flow up to 50%, while improvements up to 30% occur from placebos (Hennenberg et al. 2017; Hennenberg et al. 2014; Oelke et al. 2013; Strand et al. 2017). Due to disappointing efficacies combined with inappropriate adverse events, discontinuation rates may amount up to 70% for α_1-blockers, 83% for anticholinergics, and 91% for combination therapies in BPH within 12 months after first prescription, resulting in disease progression, complications, hospitalization, and surgery (Cindolo et al. 2015a, b; Sexton et al. 2011). Moreover, the simultaneous application of α_1-blockers and anticholinergics is still afflicted by a presumed risk to experience acute urinary retention (Drake et al. 2017). Consequently, novel options with higher efficacy and single compounds addressing the prostate and urinary bladder at once are of high demand.

It is assumed that the limited efficacy of α_1-blockers is caused by non-adrenergic mediators of prostate smooth muscle contraction (Hennenberg et al. 2013, 2017; Yu et al. 2019b). Thus, thromboxane A_2 and endothelin-1 may keep prostate tone and urethral obstruction despite treatment with α_1-blockers (Hennenberg et al. 2014, 2017). In the bladder, the origin of OAB-related detrusor contractions is neither cholinergic, nor of neurogenic origin, although anticholinergics are applied for treatment of storage symptoms (Akino et al. 2008; Kushida and Fry 2015; Nambiar et al. 2018). Hence, novel compounds completing the effects of α_1-blockers by inhibition of non-adrenergic contractions in the prostate and of cholinergic and non-cholinergic contractions in the bladder may be attractive candidates to overcome the limitations of current medical options for treatment of mixed LUTS.

The serine/threonine kinase 16 (STK16) is a poorly characterized kinase, which has been suggested with several functions, including regulation of actin organization and cell adhesion (Ligos et al. 2002; Liu et al. 2017). As actin organization and cellular adhesion are required for smooth muscle contraction (Hennenberg et al. 2014), we speculated that inhibitors for STK16 may interfere in smooth muscle contraction of the lower urinary tract. A small molecule inhibitor with assumed specificity for STK16, STK16-IN-1, has been recently developed (Liu et al. 2016). To the best of our knowledge, its effects on smooth muscle contraction have not been examined to date. Here, we studied effects of STK16-IN-1 on contractions of tissues from the human prostate and male detrusor.

Methods

Human prostate and bladder tissues

Human prostate tissues were obtained from patients who underwent radical prostatectomy for prostate cancer ($n = 68$). Patients with previous transurethral resection of the prostate were excluded. Human detrusor tissues were obtained from male patients who underwent radical cystectomy for bladder cancer ($n = 38$). This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and has been approved by the ethics committee of the Ludwig-Maximilians University, Munich, Germany (approval numbers 17-106, 19-735). Informed consent was obtained from all patients. All samples and data were collected and analyzed anonymously. Following removal of bladders or prostates from patients, macroscopic pathologic examination and sampling were performed within approximately 30 min. Organ bath studies were started within 1 h following sampling, i.e., approximately 1.5 h following surgical removal of the organs. For transport and storage, organs and tissues were stored in Custodiol® solution (Köhler, Bensheim, Germany). For macroscopic examination and sampling of prostate tissues, the prostate was opened by a single longitudinal cut reaching from the capsule to the urethra. Subsequently, both intersections were checked macroscopically for any obvious tumor infiltration. Tissues were taken solely from the periurethral zone, considering the fact that most prostate cancers arise in the peripheral zone (Pradidarcheep et al. 2011; Shaikhibrahim et al. 2012). In fact, tumor infiltration in the periurethral zone (where sampling was performed) was very rare (found in less than 1% of prostates). Prostates showing tumors in the periurethral zone upon macroscopic inspection were not subjected to sampling and were not included in this study. BPH is present in ca. 80% of patients with prostate cancer (Alcaraz et al. 2009; Orsted and Bojesen 2013). For macroscopic examination and sampling of detrusor tissues, the bladder was opened by cutting from the bladder outlet to the bladder dome. Subsequently, the intravesical surface and bladder wall were checked macroscopically for tumor infiltration. Tissues were taken from the lateral bladder wall, provided that tumor burden in the bladder wall allowed sampling. Urothelial layers were removed from samples, so that only detrusor tissues were used for experiments.

Western blot analysis

Frozen tissues were homogenized in a buffer containing 25 mM Tris/HCl, 10 μM phenylmethanesulfonyl fluoride, 1 mM benzamidine, and 10 μg/ml leupeptine hemisulfate, using the FastPrep®-24 system with matrix A (MP Biomedicals, Illkirch, France). After centrifugation (20,000g,

4 min), supernatants were assayed for protein concentration using the Dc-Assay kit (Biorad, Munich, Germany) and 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, and proteins were blotted on Protran® nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were blocked with phosphate-buffered saline (PBS) containing 5% milk powder (Roth, Karlsruhe, Germany) overnight and incubated with rabbit anti-STK16 (H-008576) (Abnova, Taipei City, Taiwan), rabbit anti-STK16 (catalog number 133967) (US Biological, Salem, MA, USA), 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).

Primary antibodies were diluted in PBS containing 0.1% Tween 20 (PBS-T) and 5% milk powder. Subsequently, detection was continued using secondary biotinylated horse anti-mouse or horse anti-rabbit IgG (BA-1000, BA-2000) (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. Membranes were washed with PBS-T after any incubation with primary or secondary antibodies or biotin-HRP. Finally, blots were developed with enhanced chemiluminescence (ECL) using ECL Hyperfilm (GE Healthcare, Freiburg, Germany). Intensities of resulting bands for STK16, PSA, and β-actin were quantified densitometrically using "ImageJ" (National Institutes of Health, Bethesda, MD, USA), and ratios between values (negative decadic logarithms of arbitrary, densitometric units) for STK/β-actin were plotted against ratios between PSA/β-actin and subjected to Spearman's correlation analysis using GraphPad Prism 6 (Statcon, Witzenhausen, Germany).

Immunofluorescence

Human prostate specimens, embedded in optimal cutting temperature (OCT) compound, were snap-frozen in liquid nitrogen and kept at $-80\,^{\circ}C$. Sections (8 μm) were cut in a cryostat and collected on Superfrost® microscope slides. Sections were post-fixed in methanol at $-20\,^{\circ}C$ and blocked in 1% bovine serum albumin before incubation with primary antibody overnight at room temperature. For double labelling, the following primary antibodies were used: rabbit anti-STK16 (H-008576) (Abnova, Taipei City, Taiwan), rabbit anti-STK16 (catalog number 133967) (US Biological, Salem, MA, USA), 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). Binding sites were visualized using Cy3-conjugated goat anti-mouse IgG (AP124C) (Millipore, Billerica, MA, US) and Cy5-conjugated goat anti-rabbit IgG (ab6564) (Abcam, Cambridge, UK). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole-dihydrochloride (DAPI) (Invitrogen, Camarillo, CA, USA). Immunolabeled sections were analyzed using a laser scanning microscope (Leica SP2, Wetzlar, Germany). Fluorescence was recorded with separate detectors. Control stainings without primary antibodies did not yield any signals.

Tension measurements

Prostate and detrusor strips $(6 \times 3 \times 3$ mm) were mounted in 10 ml aerated (95% O_2 and 5% CO_2) tissue baths (Danish Myotechnology, Aahus, Denmark) with four chambers, containing Krebs-Henseleit solution (37 °C, pH 7.4). Preparations were stretched to 4.9 mN and left to equilibrate for 45 min. In the initial phase of the equilibration period, spontaneous decreases in tone are usually observed. Therefore, tension was adjusted three times during the equilibration period, until a stable resting tone of 4.9 mN was attained. After the equilibration period, maximum contraction induced by 80 mM KCl was assessed. Subsequently, chambers were washed three times with Krebs-Henseleit solution for a total of 30 min, and STK16-IN-1 (final concentration 1 μM) or dimethylsulfoxide (DMSO) for controls were added. Cumulative concentration response curves for contractile agonists were constructed 30 min after addition of inhibitors or DMSO. Effects of STK16-IN-1 and DMSO were examined in experiments using samples from the same prostate in each experiment. Thus, from each prostate, samples were allocated to the control and inhibitor groups within the same experiment. Consequently, both groups in each series had identical group sizes. Application of solvent (two chambers) and inhibitor (two chambers) to chambers was changed for each experiment. As two chambers were used for controls and two others for inhibitors in each experiment, all values of one independent experiment were determined in duplicate. Only one curve was recorded with each sample. For calculation of agonist-induced contractions, tensions were expressed as percentage of 80 mM KCl-induced contractions, as this may correct different stromal/epithelial ratios, different smooth muscle content, varying degrees of BPH, or any other heterogeneity between prostate samples and patients. For series, where differences between the DMSO and STK16-IN-1 groups were observed, E_{max} and EC_{50} values were calculated by curve fitting for each single experiment using GraphPad Prism 6 (Statcon, Witzenhausen, Germany) and analyzed as described below.

Data and statistical analysis

Data in concentration response curves and for E_{max} and pEC_{50} values are presented as means ± standard deviation (SD) with the indicated number (n) of independent experiments. Effects of STK16-IN-1 on E_{max} and pEC_{50} values and for each agonist concentration (where an inhibition of contraction was assumed) are presented as mean differences (MD, calculated as described below) with 95% confidence interval (CI). The present study and analyses were designed to be exploratory but not designed to test a pre-specified statistical null hypothesis. Despite the exploratory design of this study, the (minimum) number of experiments and group sizes in organ bath experiments was pre-planned as $n = 5$/group. Thus, all groups were based on five or more independent experiments and included tissues from five or more patients in each group. Data were extracted and analyzed, after at least five experiments of a series were performed. Following this analysis, series were discontinued if no effect was expected on this basis, or if an inhibition was observed after these experiments. If the initial results were unclear, but suggested that an inhibitory effect may be expected, series were continued. In line with the exploratory character of our study, with our procedures and with recent recommendations for data analysis (Motulsky 2014), application of statistical tests and reporting of p values (i.e., hypothesis-testing p values, but also descriptive p values) were omitted. An exception are Spearman's correlation analyses (without p values), which were performed using GraphPad Prism 6 (Statcon, Witzenhausen, Germany). In experiments based on antibody-based detection, case numbers were adapted to technical settings (prostates, lanes in gel electrophoresis), or to availability of tissues (detrusor tissues). According to the paired design (allocation of samples from each tissue to the control and inhibitor groups), groups being compared with each other showed identical group sizes. No data or experiments were excluded from analyses, with the exception of 2 single experiments (in 2 different series, as indicated in results), where the curves did not allow automatic curve fitting by GraphPad Prism, so that calculation of E_{max} and EC_{50} values was not possible for these single experiments. Differences of E_{max} and pEC_{50} values were calculated for each single experiment (i.e., the Δ between inhibitor and control group, of corresponding, paired samples from the same prostate in each single experiment, provided that curve fitting for both groups was possible) and are expressed as MD with 95% CI, both calculated using the SPSS® version 20 (IBM SPSS Statistics, IBM Corporation, Armonk, NY, USA). Similarly, differences in agonist-induced contractions were calculated for each single experiment (i.e., for each applied agonist concentration between inhibitor and control group, for corresponding, paired samples from the same prostate in each single experiment, provided that a possible inhibition was seen in concentration response curves), and are expressed as MD with 95% CI.

Materials, drugs, and nomenclature

STK16-IN-1 (1-(4-fluoro-3-methylphenyl)-1,7-dihydro-2H-pyrrolo[2,3-h]-1,6-naphthyridin-2-one) is an inhibitor with assumed selectivity for STK16. Phenylephrine ((R)-3-[-1-hydroxy-2-(methylamino)ethyl]phenol) and methoxamine (α-(1-aminoethyl)-2,5-dimethoxybenzyl alcohol) are selective agonists for α_1-adrenoceptors. U46619 ((Z)-7-[(1S,4R,5R,6S)-5-[(E,3S)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid) is an analogue of TXA_2 and frequently used as an agonist for TXA_2 receptors. Metacholine (2-acetoxypropyl)trimethylammonium) and carbachol (2-hydroxyethyl)trimethylammonium chloride carbamate) are selective agonists for muscarinic acetylcholine receptors. STK16-IN-1 was dissolved in DMSO (10 mM), and solutions were stored at −20 °C until used. Noradrenaline, phenylephrine, methoxamine, metacholine, and carbachol were dissolved in water, and solutions were freshly prepared before each experiment. Endothelin-1 was dissolved in water, and solutions were stored at −20 °C until used. U46619 was dissolved in ethanol, and solutions were stored at −20 °C until used. STK16-IN-1 and U46619 were obtained from Tocris (Bristol, UK), noradrenaline, phenylephrine, methoxamine, metacholine, and carbachol from Sigma (Munich, Germany), and endothelin-1 from Enzo Life Sciences (Lörrach, Germany).
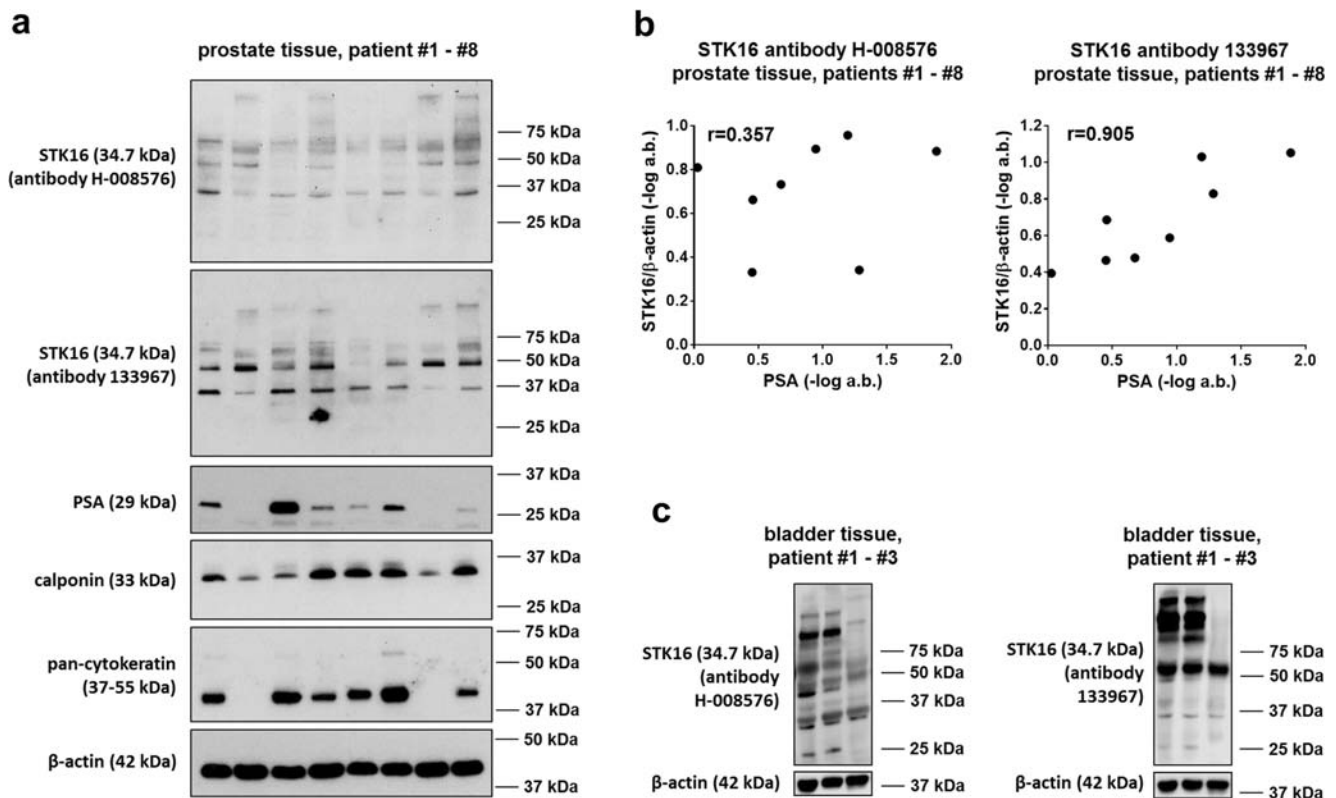
Results

Western blot analyses with anti-STK16 antibodies

Western blot analyses of human prostate tissues were performed using two different antibodies raised against STK16 (antibodies H-008576, Abnova, and 133967, US Biological). Both antibodies revealed bands around 35 kDa, i.e., with sizes matching the molecular weight of STK16 (34.7 kDa) (Fig. 1a). These bands occurred for all samples, although with different intensity (Fig. 1a). Additional, prominent bands were observed using both antibodies, which migrated at 50 kDa and around 70 kDa for both antibodies (Fig. 1a).

Correlation analyses were performed between intensities of the 35-kDa bands from STK16 detection and PSA bands (each from the same samples). A positive correlation was revealed using the STK16 antibody 133967 ($r = 0.905$) (Fig. 1b). A possible but less-pronounced correlation was observed using the STK16 antibody H-008576 ($r = 0.357$) (Fig. 1b).

Using the same antibodies raised against STK16, Western blot analyses of male detrusor tissues revealed similar band patterns as observed for prostate tissues. Thus, both antibodies revealed bands around the expected molecular weight of STK16 (34.7 kDa) (Fig. 1c).

Fig. 1 Western blot analyses of human prostate and male detrusor tissues. Western blot analyses of human prostate (**a**, **b**) and male detrusor tissues (**c**) were performed using two different antibodies raised against STK16. In prostate samples, calponin was detected as a marker for smooth muscle content, pan-cytokeratin as a marker for (glandular) epithelial cells, PSA as marker for degree of BPH, and β-actin as control in prostate and detrusor samples. On the right of each blot, positions and sizes of marker bands are indicated. Shown are blots with all samples being included to Western blot analyses. In **b**, ratios of values from densitometric quantification for STK16/β-actin and PSA/β-actin were plotted against each other, and subjected to correlation analyses (r values from Spearman's correlation analyses in inserts)

Additional bands occurred again around 50 kDa and 75 kDa (Fig. 1c). These additional bands were of similar intensity as the bands around 37 kDa (H-008576) or stronger (133967) (Fig. 1c).

Immunofluorescence stainings with anti-STK16 antibodies

Double labeling of prostate sections was performed using two different antibodies raised against STK16, each of them together with an anti-calponin antibody for labeling of smooth muscle cells, or an anti-pan-cytokeratin antibody for labeling of glandular epithelial cells (Fig. 2). Sections of prostate tissues showed a typical architecture composed of stromal areas with immunoreactivity to the anti-calponin antibody, surrounding glands with immunoreacitivity to the anti-pan-cytokeratin antibody in the glandular epithelia (Fig. 2). Immunoreactivity for both anti-STK16 antibodies was observed in the stroma and in glandular epithelia, where it colocalized with calponin and pan-cytokeratin immunoreactivities (Fig. 2).

Effects of STK16-IN-1 on U46619-induced prostate contractions

U46619 (0.1–30 μM) induced concentration-dependent contractions of human prostate tissues (Fig. 3a). Following application of STK16-IN-1 (1 μM), U46619-induced contractions were lower than contractions observed in controls, i.e., after application of DMSO ($n = 10$) (Fig. 3a, Table 1). Inhibition of U46619-induced contractions was confirmed by calculation of E_{max} values by curve fitting, which was possible for all experiments in the DMSO group and for nine of ten experiments in the STK16-IN-1 group. Curve fitting revealed E_{max} values for U46619-induced contractions of $46.2 \pm 25.2\%$ of KCl-induced contractions following application of DMSO, and $17.7 \pm 11.3\%$ following application of STK16-IN-1 (Fig. 3a) (MD − 30.4% of KCl-induced contraction [95% CI − 45.7 to − 15.2]). Calculation of EC_{50} values by curve fitting was possible for all experiments in the DMSO group, and for nine of ten experiments in the STK16-IN-1 group, and suggested no effect of STK16-IN-1 on EC_{50} values for U46619 (Fig. 3a). Curve fitting revealed pEC_{50} values for U46619 of 7.4 ± 1.6 M following application of DMSO, and 7.1 ± 2.0 M

Fig. 2 Immunofluorescence staining of human prostate tissues for STK16. Sections were double labeled with antibodies for STK16 (two different, as indicated), together with antibodies for calponin (marker for smooth muscle cells) or pan-cytokeratin (marker for glandular epithelial cells). Yellow color in merged pictures indicates colocalization of targets. Shown are representative stainings from series with tissues from $n = 5$ patients for each combination. Negative controls were performed without primary antibodies but Cy3- and Cy5-coupled secondary antibodies and DAPI (not shown)
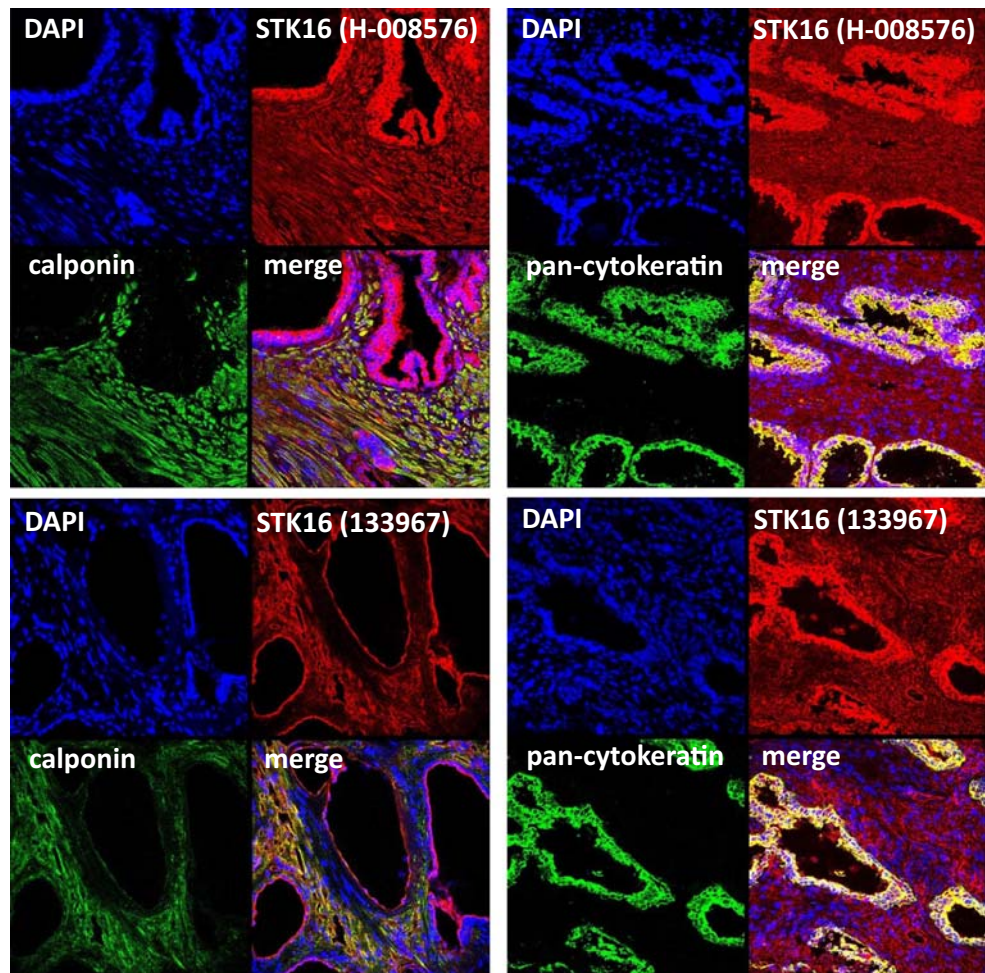
following application of STK16-IN-1 (MD − 0.5 M [95% CI − 1.7 to 0.6]).

Effects of STK16-IN-1 on endothelin-induced prostate contractions

Endothelin-1 induced similar contractions of human prostate tissues, which were only slightly concentration-dependent in the applied range of 0.1–3 μM (Fig. 3b). Following application of STK16-IN-1 (1 μM), contractions induced by endothelin-1 were lower than contractions observed in controls, i.e., after application of DMSO ($n = 12$) (Fig. 3b, Table 1). Inhibition of endothelin-1-induced contractions was confirmed by calculation of E_{max} values by curve fitting, which was possible for all experiments in the DMSO group, and for eleven of twelve experiments in the STK16-IN-1 group. Curve fitting revealed E_{max} values for endothelin-1-induced contractions of 139.3 ± 95.4% of KCl-induced contractions following application of DMSO, and 81.7 ± 47.1% following application of STK16-IN-1 (Fig. 3b) (MD − 54% of KCl-induced contraction [95% CI − 98 to − 9.5]). Calculation of EC_{50} values by curve fitting was possible for all

experiments in the DMSO group, and for eleven of twelve experiments in the STK16-IN-1 group, and suggested no effect of STK16-IN-1 on EC_{50} values for endothelin-1 (Fig. 3b). Curve fitting revealed pEC_{50} values for endothelin-1 of 7.8 ± 2.5 M following application of DMSO and 7.4 ± 2.3 M following application of STK16-IN-1 (MD − 0.9 M [95% CI − 2.2 to 0.5]).

Endothelin-2 and endothelin-3 (0.1–3 μM) induced concentration-dependent contractions of human prostate tissues (Fig. 3b, c). Prostate contractions induced by endothelin-2 and endothelin-3 were not affected by STK16-IN-1 (1 μM) (Fig. 3b, c).

Effects of STK16-IN-1 on EFS-induced and adrenergic prostate contractions

Noradrenaline, phenylephrine, and methoxamine (0.1–100 μM) induced concentration-dependent contractions of human prostate tissues (Fig. 4). STK16-IN-1 (1 μM) did not affect these contractions, so that adrenergic contractions were similar following application of STK16-IN-1 and DMSO (Fig. 4). EFS (2–32 Hz) induced frequency-

Fig. 3 Effects of STK16-IN-1 on non-adrenergic human prostate smooth muscle contraction. Contractions in an organ bath were induced by the thromboxane A_2 analog U46619 (**a**), and by endothelins (**b**–**d**), 30 min after addition of STK16-IN-1 (1 μM) or DMSO. To eliminate heterogeneities including any individual variations, different degrees of BPH, or varying smooth muscle content (compare Fig. 1), tensions have been expressed as % of high molar KCl-induced contraction, which was assessed before application of STK16-IN-1 or DMSO. In each single experiment, samples from the same tissue were used for the control and inhibitor group. Shown are means ± SD from series with tissues from n = 10 patients for U46619, n = 12 for endothelin-1, n = 6 for endothelin-2, and n = 8 for endothelin-3, E_{max} values for single experiments (calculated by curve fitting) where calculation was possible (see text), and pEC_{50} values for single experiments (calculated by curve fitting) where calculation was possible (see text)
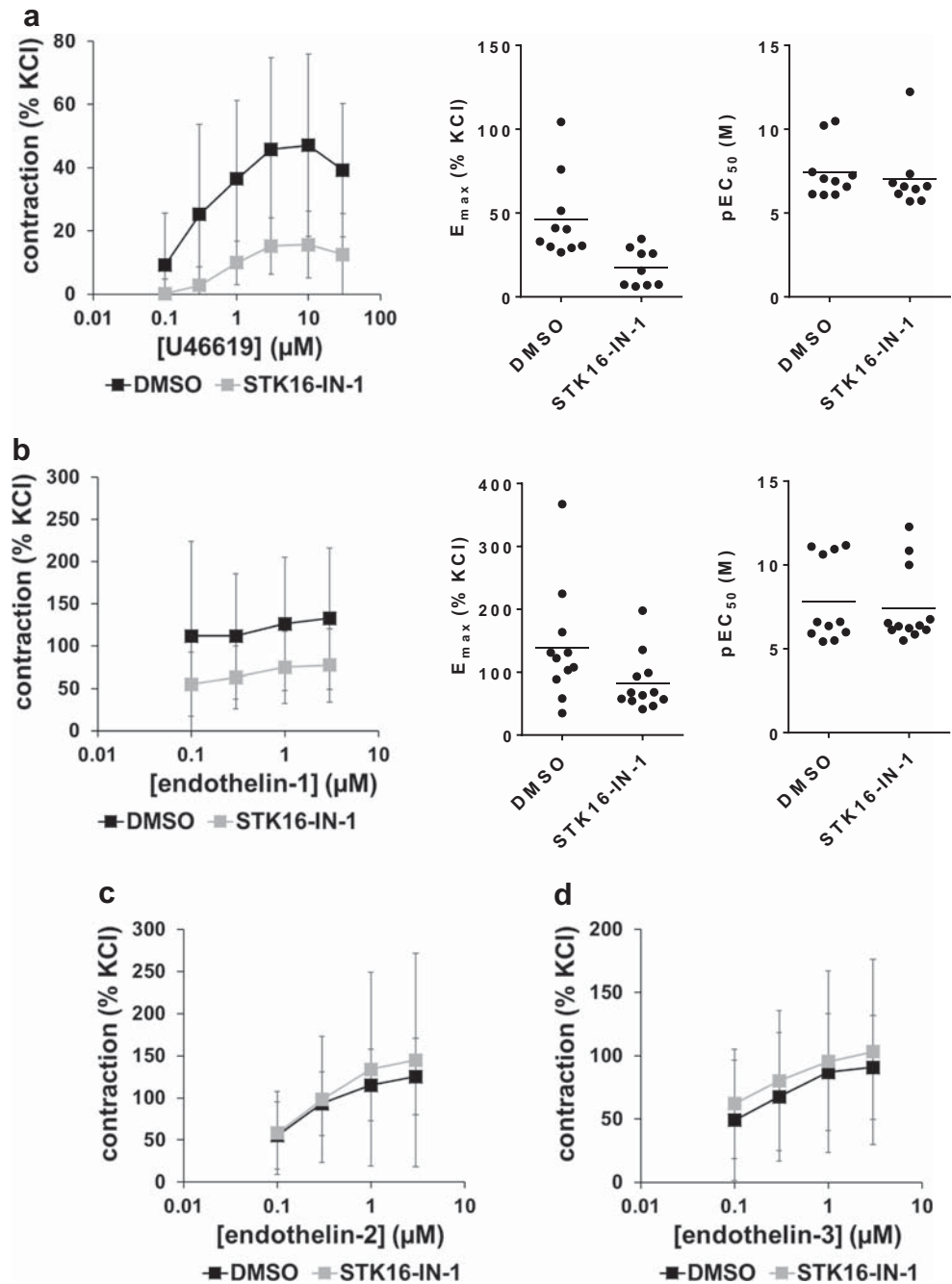
dependent contractions of human prostate tissues (Fig. 5a). STK16-IN-1 (1 μM) did not affect these contractions, so that EFS-induced contractions were similar following application of STK16-IN-1 and DMSO (Fig. 5a).

Effects of STK16-IN-1 on EFS-induced detrusor contractions

EFS (2–32 Hz) induced frequency-dependent contractions of male human detrusor tissues (Fig. 5b). Detrusor

contractions induced by EFS were not affected by STK16-IN-1, so that EFS-induced contractions were similar following application of STK16-IN-1 and DMSO (1 μM) (Fig. 5b).

Effects of STK16-IN-1 on U46619-induced detrusor contractions

U46619 (0.1–30 μM) induced concentration-dependent contractions of male human detrusor tissues (Fig. 6a). Following application of STK16-IN-1 (1 μM), U46619-

Table 1 Mean differences (MDs) for agonist-induced contractions after application of STK16-IN-1 (1 µM) or DMSO (as control), shown for each applied agonist concentration, and with 95% confidence intervals (CI) (in square brackets, low to high) (% of KCl-induced contractions). Calculations were performed for those agonists and tissues, where a possible inhibition of contraction by STK16-IN-1 was observed in concentration response curves (compare Figs. 3 and 6) (n.a., concentration not applied). Differences in contraction at given agonist concentrations were calculated for each single experiment (i.e., between inhibitor and control group, for corresponding, paired samples from the same prostate or bladder in each single experiment) and are expressed as MD with 95% CI. Data are based on experiments from series with $n = 10$ prostates and $n = 8$ bladders for U46619, $n = 12$ prostates for endothelin-1, $n = 10$ bladders for metacholine, and $n = 5$ bladders for carbachol

|		Agonist concentration						
		0.1 µM	0.3 µM	1 µM	3 µM	10 µM	30 µM	100 µM
Prostate	U46619	−9.1 [−19.9 to 1.7]	−22.4 [−40.6 to −4.2]	−26.5 [−42.1 to −10.9]	−30.6 [−48.4 to −12.9]	−31.5 [−47.5 to −15.5]	−26.6 [−37.7 to −15.6]	n.a.
	Endothelin-1	−56.6 [−110.4 to −2.8]	−48.5 [−81.4 to −15.6]	−51.5 [−84.9 to −18.1]	−55.6 [−95.2 to −15.9]	n.a.	n.a.	n.a.
Detrusor	U46619	−1.0 [−3.7 to 1.8]	−1.3 [−4.3 to 1.8]	−3.6 [−7.5 to 0.3]	−2.1 [−7.1 to 2.9]	−8.6 [−27.6 to 10.4]	−6.2 [−12.7 to 0.3]	n.a.
	Metacholine	−0.7 [−1.6 to 0.3]	−2.4 [−4.5 to −0.4]	−5.5 [−9.2 to −1.8]	−24.1 [−42.4 to −5.9]	−41.0 [−65.7 to −16.3]	−25.3 [−57.2 to 6.6]	−19.9 [−44.4 to 4.8]
	Carbachol	−15.4 [−47.8 to 16.9]	−11.3 [−51.2 to 28.5]	−26.0 [−92.5 to 40.5]	−20.1 [−74.1 to 34.0]	−27.9 [−61.4 to 5.6]	−30.1 [−55.9 to −4.4]	−23.5 [−45.2 to −1.8]

Fig. 4 Effects of STK16-IN-1 on adrenergic human prostate smooth muscle contraction. Contractions in an organ bath were induced by noradrenaline and the α_1-adrenergic agonists phenylephrine and methoxamine, 30 min after addition of STK16-IN-1 (1 μM) or DMSO. To eliminate heterogeneities including any individual variations, different degree of BPH, or varying smooth muscle content (compare Fig. 1), tensions have been expressed as % of high molar KCl-induced contraction, which was assessed before application of STK16-IN-1 or DMSO. In each single experiment, samples from the same tissue were used for the control and inhibitor group. Shown are means ± SD from series with tissues from $n = 12$ patients for noradrenaline, $n = 6$ for phenylephrine, and $n = 5$ for methoxamine
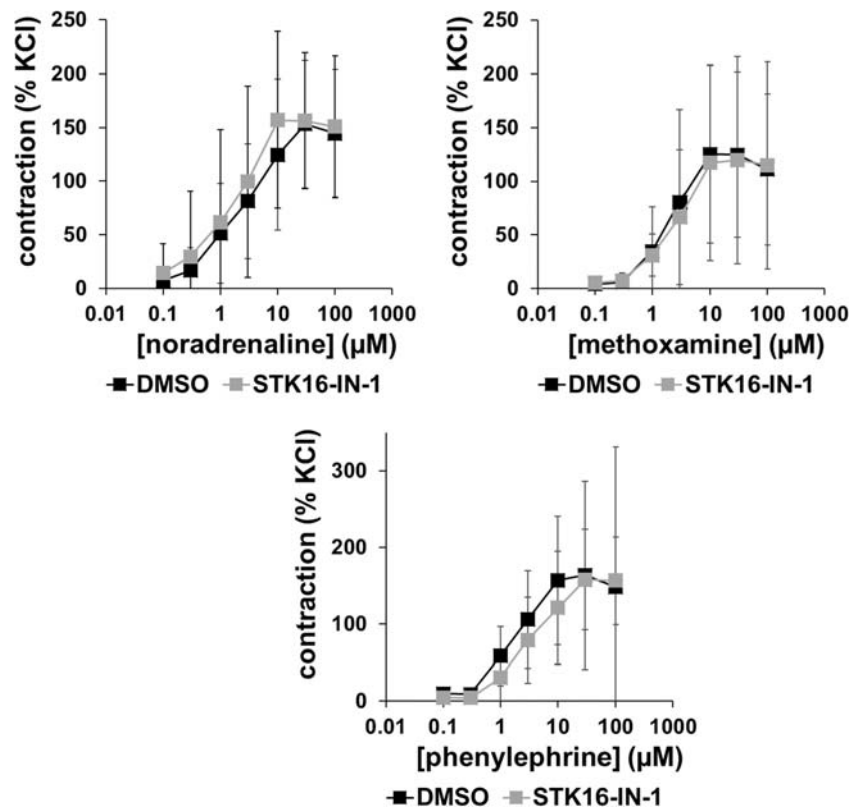
induced contractions were lower than contractions observed in controls, i.e., after application of DMSO ($n = 8$) (Fig. 6a, Table 1). This inhibition was confirmed by calculation of E_{max} values by curve fitting, which was possible for all experiments. Curve fitting revealed E_{max} values for U46619-induced contractions of $21.2 \pm 11\%$ of KCl-induced contractions following application of DMSO, and $13.8 \pm 6.6\%$ following application of STK16-IN-1 (Fig. 6a) (MD -7.4% of KCl-induced contraction [95% CI -18.5 to 3.7]). Calculation of EC_{50} values by curve fitting was possible for all experiments and suggested no effect of STK16-IN-1 on EC_{50} values for U46619. Curve fitting revealed pEC_{50} values for U46619 of 6 ± 0.7 M following application of DMSO and 6 ± 0.5 M following application of STK16-IN-1 (MD 0.04% M [95% CI -0.5 to 0.6]).
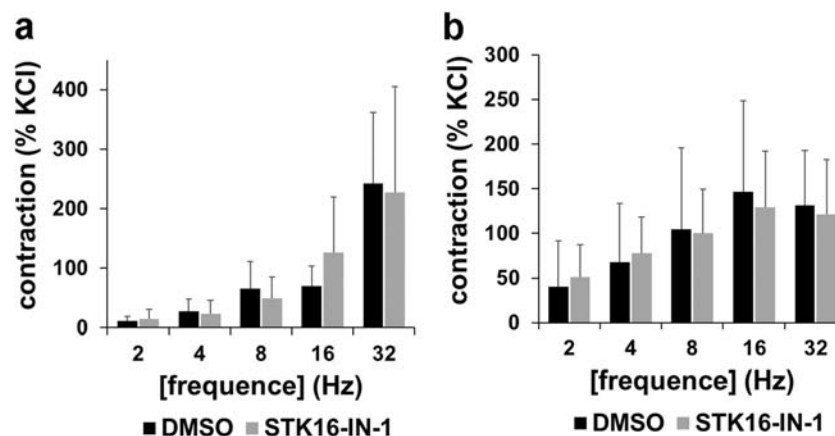
Fig. 5 Effects of STK16-IN-1 on EFS-induced contractions of human prostate and male detrusor smooth muscle. Contractions of human prostate tissues (**a**) or male human detrusor tissues (**b**) in an organ bath were induced by EFS, 30 min after addition of STK16-IN-1 (1 μM) or DMSO. To eliminate heterogeneities including any individual variations, different degree of BPH, or varying smooth muscle content (compare Fig. 1), tensions have been expressed as % of high molar KCl-induced contraction, which was assessed before application of STK16-IN-1 or DMSO. In each single experiment, samples from the same tissue were used for the control and inhibitor group. Shown are means ± SD from series with tissues from $n = 10$ patients for prostate and $n = 6$ patients for detrusor

Fig. 6 Effects of STK16-IN-1 on agonist-induced human male detrusor smooth muscle contraction. Contractions in an organ bath were induced by the thromboxane A_2 analog U46619 (**a**), and the cholinergic agonists metacholine (**b**) and carbachol (**c**), 30 min after addition of STK16-IN-1 (1 μM) or DMSO. To eliminate heterogeneities including any individual variations or varying smooth muscle content (compare Fig. 1), tensions have been expressed as % of high molar KCl-induced contraction, which was assessed before application of STK16-IN-1 or DMSO. In each single experiment, samples from the same tissue were used for the control and inhibitor group. Shown are means ± SD from series with tissues from $n = 8$ patients for U46619, $n = 10$ for metacholine, and $n = 5$ for carbachol, E_{max} values for all single experiments (calculated by curve fitting), and pEC_{50} values for all single experiments (calculated by curve fitting)
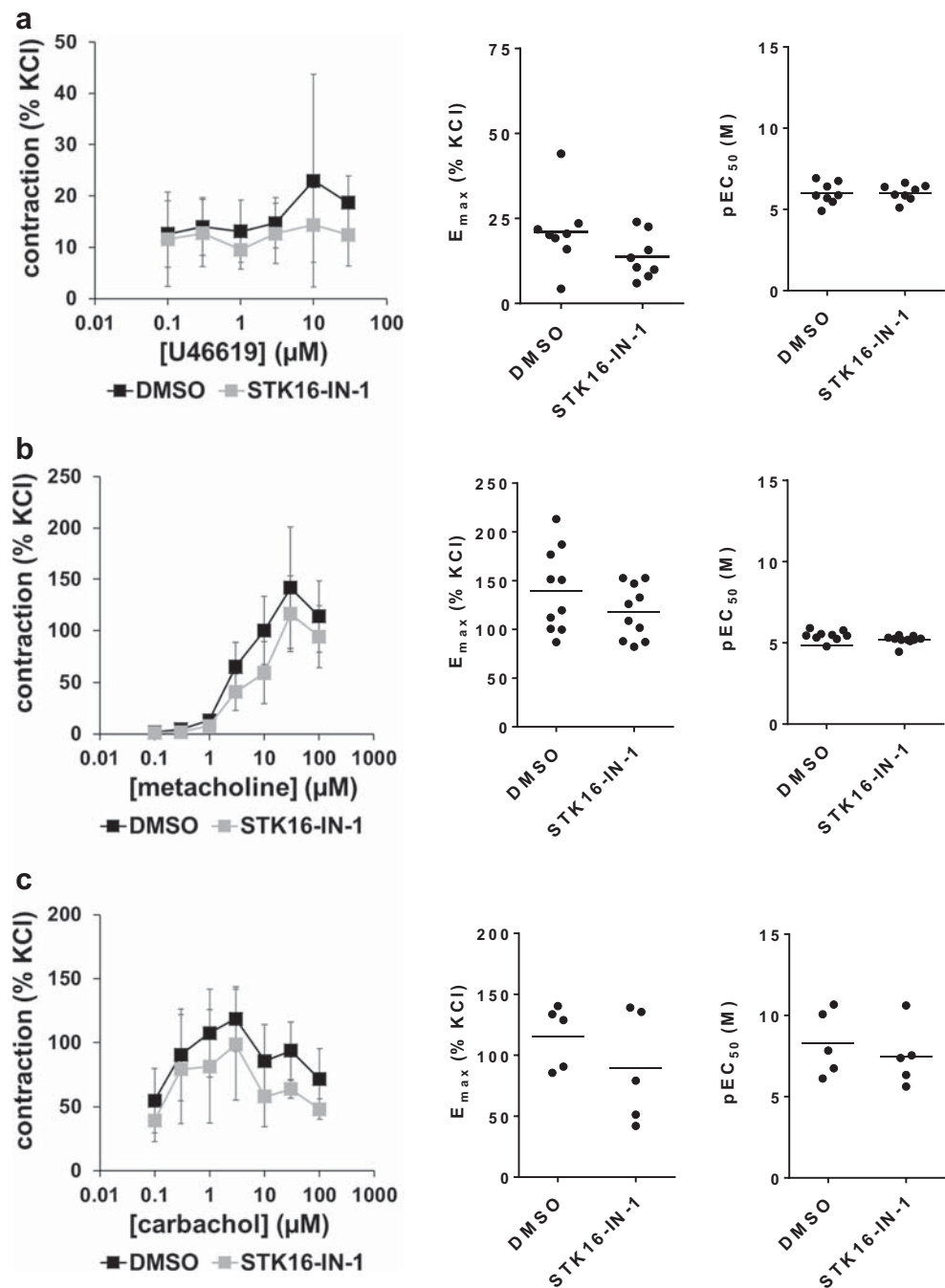
Effects of STK16-IN-1 on cholinergic detrusor contractions

Metacholine and carbachol (0.1–100 μM) induced concentration-dependent contractions of male human detrusor tissues (Fig. 6b, c). Following application of STK16-IN-1 (1 μM), contractions induced by metacholine and carbachol were lower than contractions observed in controls, i.e., after application of DMSO ($n = 10$ for metacholine, $n = 5$ for carbachol) (Fig. 6b, c, Table 1). This inhibition was confirmed for both agonists by calculation of E_{max} values by curve fitting,

which was possible for all experiments, if the concentration range of 0.1–10 μM was considered. Curve fitting revealed E_{max} values for metacholine-induced contractions of 139.8 ± 42.6% of KCl-induced contractions following application of DMSO, and 117.8 ± 28% following application of STK16-IN-1 (Fig. 6b) (MD − 21.9% of KCl-induced contraction [95% CI − 45.2 to 1.3]). Calculated E_{max} values for carbachol-induced contractions were 115.8 ± 25.7% of KCl-induced contractions following application of DMSO, and 89.4 ± 45.9% following application of STK16-IN-1 (Fig. 6c) (MD − 26.4% of KCl-induced contraction [95% CI − 78.3 to 25.4]). Calculation of

EC_{50} values by curve fitting was possible for all experiments, if the concentration range of 0.1–10 μM was considered, and suggested no effect of STK16-IN-1 on EC_{50} values for metacholine or carbachol. Curve fitting revealed pEC_{50} values for metacholine of 4.9 ± 1.9 M following application of DMSO and 5.2 ± 0.3 M following application of STK16-IN-1 (MD 0.3 M [95% CI − 1.0 to 1.7]), and pEC_{50} values for carbachol of 8.3 ± 2 M following application of DMSO and 7.5 ± 1.9 M following application of STK16-IN-1 (MD − 0.8 M [95% CI − 3.4 to 1.8]) (Fig. 6b, c).

Discussion

The principal aim of the present study was to examine the effects of an STK16 inhibitor on smooth muscle contractions of human prostate and male detrusor tissues. Prostate and detrusor smooth muscle contraction are of high clinical interest in urology, (1) as they may contribute to LUTS suggestive of BPH and/or OAB, and (2) because inhibition of these contractions is an important strategy for medical treatment of LUTS (Hennenberg et al. 2014; Oelke et al. 2013). The basis to assume any effects of STK16 inhibitors on smooth muscle contractions was provided by recent reports suggesting a role of STK16 for promotion of actin organization and for control of cell adhesion (Ligos et al. 2002; Liu et al. 2017; Lopez-Coral et al. 2018), which are in turn required for smooth muscle contraction (Hennenberg et al. 2014). With STK16-IN-1, a small molecule inhibitor with assumed specificity for STK16 is now available (Liu et al. 2016). Our main findings suggest that STK16-IN-1 inhibited non-adrenergic (i.e., thromboxane A_2- and endothelin-1-induced) contractions of prostate smooth muscle, and thromboxane A_2-induced and (to limited extent) cholinergic contractions of detrusor smooth muscle.

From a clinical point of view, in particular, the inhibition of non-adrenergic contractions in the prostate may be promising. Inhibition of adrenergic contractions is possible by administration of α_1-blockers, which are the first-line option for medical treatment of LUTS suggestive of BPH (Oelke et al. 2013). It is widely assumed that α_1-blockers may improve symptoms by relaxation of prostate smooth muscle, followed by enhanced urinary flow and bladder emptying (Hennenberg et al. 2014; Oelke et al. 2013). Despite their high popularity, their effects are clearly limited to improvements of urinary flow or of perceived symptoms (international prostate symptom score, IPSS) of 50%, while even placebos may cause improvements up to 27% (Hennenberg et al. 2014, 2017; Oelke et al. 2013; Strand et al. 2017). It has been suggested that the limited efficacy of α_1-blockers is caused by non-adrenergic prostate smooth muscle contractions, being induced by endothelin-1 and/or thromboxane A_2 (Hennenberg et al. 2014, 2017). Thus, these non-adrenergic mediators may keep prostate smooth muscle tone, and therefore bladder

outlet obstruction despite therapy with α_1-blockers (Hennenberg et al. 2014, 2017). Consequently, compounds inhibiting the non-adrenergic prostate smooth muscle contractions may be attractive candidates to overcome the current limitations of α_1-blockers. STK16-IN-1 inhibited thromboxane A_2- and endothelin-1-induced contractions of human prostate tissues, which may qualify STK16-IN-1 as such a candidate compound.

Therapy of male LUTS was restricted to treatment of obstructive symptoms suggestive of BPH for decades. It is now clear that many patients simultaneously show symptoms due to OAB in addition to LUTS suggestive of BPH, referred to as mixed LUTS (Fullhase et al. 2013; Oelke et al. 2013). Consequently, the use of anticholinergics has been considered for male LUTS. Initial concerns that combined treatment with α_1-blockers (first-line option for treatment of LUTS suggestive of BPH) and anticholinergics (first-line option for treatment of OAB) may increase the risk for urinary retention did not prove true (Drake et al. 2017). Nevertheless, these combinations are still not routinely applied, so that medical therapy of male LUTS may be still focussed on BPH and remains a challenge. Thus, compounds inhibiting cholinergic and non-cholinergic detrusor contractions, and simultaneously completing the effects of α_1-blockers in the prostate (i.e., inhibiting non-adrenergic prostate contractions) may be interesting candidates for in vivo studies addressing mixed LUTS. Although the effect of STK16-IN-1 on detrusor contractions was probably too small to expect adequate improvements of storage symptoms in vivo, the current findings may suggest that compounds with the properties explained above may exist.

STK16-IN-1 has been reported to inhibit STK16 with high selectivity and potency (Liu et al. 2016). The properties of this compound were identified by profiling of a kinase inhibitor library (Liu et al. 2016). From a total of 442 tested kinases, only STK16 and mTOR were potently inhibited (> 99% inhibition of both kinases) using STK16-IN-1 in a concentration of 10 μM (Liu et al. 2016). Biochemical assays revealed IC_{50} values of STK16-IN-1 of 0.295 μM for STK16 and of 5.56 μM for mTOR and 0.856 μM to 1.070 μM for phosphoinositide 3-kinase (PI3K) isoforms α, γ, and δ (Liu et al. 2016). In cultured cells, EC_{50} values were higher than IC_{50} values in biochemical assays, and inhibition of PI3K substrates was not observed even using 10 μM of STK16-IN-1 (Liu et al. 2016). Consequently, a high specificity of STK16-IN-1 for STK16 has been assumed (Liu et al. 2016). Considering these findings, it appears likely that the effects observed using 1 μM of STK16-IN-1 in our organ bath experiments were attributed to inhibition of STK16, but probably not caused by off-target effects. In cell culture, 10 μM of STK16-IN-1 inhibited the proliferation of several cancer cell lines, although to low degree (Liu et al.

2016). To the best of our knowledge, other studies reporting effects of STK16 inhibitors are not available.

STK16 belongs to the family of Numb-associated kinases (NAKs). Its functions and occurrence are poorly understood. Suggested functions include actin organization, secretion, regulation of transcription factors, cell cycle progression, and cell adhesion control (Eswaran et al. 2008; Guinea et al. 2006; In et al. 2014; Li et al. 2018; Ligos et al. 2002; Liu et al. 2017; Long et al. 2013; Lopez-Coral et al. 2018; Stairs et al. 2005; Wang et al. 2019b). A proteome analysis comparing the expression of all known genes between 32 different human tissues demonstrated that STK16 was expressed in all examined tissue types (Uhlen et al. 2015). This study included tissues from four prostates and from two urinary bladders (genders not indicated), where the variation of expression levels in both organs appeared rather low compared to larger variations observed for other organs (Uhlen et al. 2015). Expression levels in the lower urinary tract were close (prostate) or higher (bladder) to the overall average level calculated for all organs; however, low case numbers may limit these conclusions (Uhlen et al. 2015). Previously, mRNA level in human tissues was reported to be highest in the kidney, pituitary gland, ovary, and testis, but also high in the pancreas, heart, liver, and colon, whereas lower urinary tract tissues were obviously not included to this analysis (Ohta et al. 2000). In fact, STK16 has been supposed to show an ubiquitous distribution, with high expression levels at least in the liver and kidney (Wang et al. 2019a). In adult mice, mRNA levels of STK16 were highest in the liver, kidney, and testis (Kurioka et al. 1998; Ligos et al. 2002), or in the mammary gland, ovary, liver, kidney, and small intestine in another study (Stairs et al. 1998). Levels may differ between different cell types within the same organ (Stairs et al. 1998; Wang et al. 2019a). In prostates from adult mice, STK16 mRNA was detectable in stromal and epithelial cells, but higher in the latter (Stairs et al. 1998).

In addition to our findings from organ bath experiments, the possibility of an expression in smooth muscle cells of the human prostate and male detrusor may be supported by our findings from Western blots analysis. Thus, two different antibodies raised against STK16 revealed bands with sizes matching the expected molecular weight of STK16, so that these may reflect STK16. In fact, previous immunoblot analyses of cells transfected with full STK16 revealed bands of this size, while no bands were observed at cells with empty vectors (Kurioka et al. 1998; Stairs et al. 1998). A positive correlation of the intensity of presumed STK16 bands with PSA content appears possible on the basis of our Western blot data. As PSA increases with the degree of BPH (Levitt and Slawin 2007), STK16 expression may increase with BPH. However, validation data for these antibodies are lacking. As both antibodies revealed further bands in addition to those matching the size of STK16, our results from fluorescence stainings of prostate tissues, where immunoreactivity

colocalized with calponin in smooth muscle cells, may be of limited value. As additional bands were strongest in analyses of detrusor tissues, we did not perform stainings of bladder samples.

Receptor-induced smooth muscle contraction has been attributed to activation of intracellular signaling pathways, which are shared by different contractile pathways and organs. In particular, the calcium-, protein kinase C-, and RhoA/Rho kinase-dependent pathways are most widely accepted and mediate adrenergic, cholinergic, and non-adrenergic/non-cholinergic contractions in the cardiovascular system, airways, lower urinary tract, and gastrointestinal tract (Somlyo and Somlyo 2000, 2003). For prostate smooth muscle, it became recently obvious, that contractions of some receptors are regulated by receptor-selective pathways, which are not shared by all contractile receptors, as several inhibitors interferred with adrenergic, but not the non-adrenergic contractions of prostate smooth muscle (Hennenberg et al. 2018; Wang et al. 2016; Yu et al. 2018, a, b). Obviously, an inverse situation applies for a STK16-IN-1-sensitive pathway, as U46619- and endothelin-1-induced, but not the adrenergic contractions were susceptible to STK16-IN-1. The molecular mechanisms underlying the agonist-selective inhibition of contraction by STK16-IN-1, and the possible role of actin organization, still remain a subject of further studies. Together, our findings show that adrenergic and non-adrenergic contractions in the lower urinary tract are divergently regulated, at least in the prostate, by a STK16-IN-1-sensitive mechanism. Notably, this divergent regulation even applies for the different endothelin isoforms. Finally, any explanation why STK16-IN-1 inhibited detrusor contractions induced by cholinergic agonists (at least to some extent), but not neurogenic contractions, remains speculation at the present stage. In fact, neurogenic contractions are at least partially mediated by cholinergic neurotransmission (Andersson 2011; Andersson and Arner 2004). This may show that smooth muscle contraction and its regulation in the lower urinary tract are more complex than assumed. Further mediators or regulators of detrusor contraction, which were not examined here or are even unknown, may be involved in this discrepant finding.

Together, our findings may be interesting from a dual perspective. From a clinical point of view, compounds addressing non-adrenergic contractions in the lower urinary tract appear promising, as they may complete the effects of α_1-blockers or anticholinergics. This may be an attractive perspective to overcome the limitations of currently available medications, and of present challenges associated with the treatment of mixed LUTS. Thus, in vivo studies with STK16-IN-1 appear feasible, as urodynamic effects appear possible. From a view of basic research, our findings point to a divergent regulation of receptor-induced smooth muscle contractions in the lower urinary tract, which is imparted by a STK16-IN-1-sensitive mechanism.

Conclusions

STK16-IN-1 inhibits non-adrenergic smooth muscle contractions in the human prostate and to limited extent in the male detrusor. In vivo studies with STK16-IN-1 appear feasible, as urodynamic effects appear possible, which may include voiding and possibly storage symptoms. The selective effect of STK16-IN-1 on adrenergic and non-adrenergic contractions points to a divergent regulation of receptor-induced smooth muscle contractions in the lower urinary tract, which is imparted by a STK16-IN-1-sensitive mechanism.

Compliance with ethical standards

Conflict of interest   The authors declare that they have no conflict of interest.

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