Life Sciences 308 (2022) 120931

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Permixon®, hexane-extracted *Serenoa repens*, inhibits human prostate and bladder smooth muscle contraction and exerts growth-related functions in human prostate stromal cells

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ARTICLE INFO

Keywords:
Lower urinary tract symptoms
Overactive bladder
Phytotherapy
Plant extracts
Prostatic hyperplasia
Smooth muscle contraction

ABSTRACT

Aims: Recently, the European Association of Urology recommended hexane-extracted fruit of *Serenoa repens* (HESr) in their guidelines on management of non-neurogenic male lower urinary tracts symptoms (LUTS). Despite previously lacking recommendations, Permixon® is the most investigated HESr in clinical trials, where it proved effective for male LUTS. In contrast, underlying mechanisms were rarely addressed and are only marginally understood. We therefore investigated effects of Permixon® on human prostate and detrusor smooth muscle contraction and on growth-related functions in prostate stromal cells.

Main methods: Permixon® capsules were dissolved using n-hexane. Contractions of human prostate and detrusor tissues were induced in organ bath. Proliferation (EdU assay), growth (colony formation), apoptosis and cell death (flow cytometry), viability (CCK-8) and actin organization (phalloidin staining) were studied in cultured human prostate stromal cells (WPMY-1).

Key findings: Permixon® inhibited α_1-adrenergic and thromboxane-induced contractions in prostate tissues, and methacholine-and thromboxane-induced contractions in detrusor tissues. Endothelin-1-induced contractions were not inhibited. Neurogenic contractions were inhibited in both tissues in a concentration-dependent manner. In WPMY-1 cells, Permixon® caused concentration-dependent breakdown of actin polymerization, inhibited colony formation, reduced cell viability, and proliferation, without showing cytotoxic or pro-apoptotic effects.

Significance: Our results provide a novel basis that allows, for the first time, to fully explain the ubiquitous beneficial effects of HESr in clinical trials. HESr may inhibit at least neurogenic, α_1-adrenergic and thromboxane-induced smooth muscle contraction in the prostate and detrusor, and in parallel, prostate stromal cell growth. Together, this may explain symptom improvements by Permixon® in previous clinical trials.

1. Introduction

Lower urinary tract symptoms (LUTS) consist of both voiding and storage symptoms [1,2]. Urethral obstruction leading to voiding symptoms is most commonly attributed to benign prostatic hyperplasia (BPH), where hyperplastic growth and increased smooth muscle tone in

Abbreviations: 5-AR, 5α-reductase; 5-ARI, 5α-reductase inhibitor; 7-AAD, 7-aminoactinomycin D; APC, allophycocyanin; ATCC, American Type Culture Collection; BPH, benign prostatic hyperplasia; CCK-8, cell counting kit 8; DHT, dihydrotestosterone; EAU, European Association of Urology; EdU, 5-ethynyl-2′-deoxyuridine; ET_A, endothelin receptor A; ET_B, endothelin receptor B; FCS, fetal calf serum; HeSr, Hexane-extracted *Serenoa repens*; LUTS, lower urinary tract symptoms; OAB, overactive bladder; OD, optical density; RCT, randomized controlled trial; RPMI, Roswell Park Memorial Institute; WPMY-1, immortalized human prostate stromal cell line (benign).

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https://doi.org/10.1016/j.lfs.2022.120931
Received 22 July 2022; Received in revised form 25 August 2022; Accepted 1 September 2022
Available online 6 September 2022

the hyperplastic prostate may lead to bladder outlet obstruction (BOO) [1,3]. Spontaneous contractions of the detrusor muscle may cause storage symptoms, which are referred to as overactive bladder (OAB) [2,4]. With a considerable proportion of patients suffering from "mixed LUTS", a combination of voiding and storage symptoms, LUTS affect a large portion of the population worldwide [1,3]. In 2018, an estimated 2.7 billion patients suffered from storage symptoms and 1.1 billion patients suffered from voiding symptoms [5]. In both BPH and OAB, exaggerated smooth muscle tone are important targets of medical therapy [1,3,4]. While smooth muscle contraction in the human prostate and detrusor is induced through agonist-activated contractile receptors, hyperplastic prostate growth depends on growth factors and hormones, such as dihydrotestosterone (DHT), and reduction of testosterone is catalyzed by 5α-reductase (5-AR) into its biologically more active metabolite DHT [3,6].

Standard-of-care treatment for LUTS/BPH includes α_1-adrenoceptor antagonists (α_1-blockers) for reduction of prostate smooth muscle tone, and 5-AR inhibitors (5-ARI) for reduction of prostate size [1]. Alpha$_1$-adrenoceptor antagonists improve prostate symptom scores (IPSS) and urinary flow rates (Q_{max}) by 50 %, while 5-ARI reduce prostate size up to 25 %. Side effects of α_1-blockers are mostly cardiovascular, and include hypotension, dizziness and a tendency to fall, whereas 5-ARI use may lead to bothersome heat waves, loss of libido, or even a combination of sexual dysfunction and depression, subsumed under the term post-finasteride-syndrome [1,7,8]. Medical therapy for LUTS/OAB includes muscarinic receptor antagonists for relief of spontaneous bladder contractions [1,2]. While antimuscarinics may improve storage symptoms in up to 65 % of patients, side effects include dry mouth, obstipation, tachycardia, and may even lead to cognitive impairment [9,10]. The more recent introduction of the β_3-adrenoceptor agonist, mirabegron, for treatment of storage symptoms reflects the need for new substance classes for medical therapy of male LUTS [1,11]. However, the specificity of mirabegron for β_3-adrenergic receptors has been increasingly questioned [12], as the compound antagonizes α_1-adrenoceptors, at least at high concentrations, out of therapeutic range [12–14]. Recently, clinical trials have increasingly focused on combination therapies, reflecting on the increasing amount of patients suffering from mixed LUTS [1,3]. By combining α_1-blockers with muscarinic receptor antagonists, two main pharmacological processes are targeted at once. However, adverse events of both drug classes are seen with combined treatment using α_1-blockers and antimuscarinics, with the most common and bothersome side-effects including ejaculation failure and therapy-limiting dry mouth [15]. Thus, respective discontinuation rates of up to 90 % due to treatment failure or therapy-limiting side effects highlight the limitations of current pharmacotherapy and may explain patients' desire for over-the-counter phytotherapeutic agents [7,9].

Phytotherapeutic agents are part of a growing market, as patients seek alternatives to current standard-of-care treatment. Most phytotherapeutic agents are available without prescription, so that their access does not warrant a urologist's appointment, which can be bothersome and time consuming [16]. However, potential mechanisms of action for phytotherapeutic agents remain unclear. There is a wide variety of plant preparations, including extracts from different species and different parts of plants, which are available as mono-preparations or as preparations combining different extracts in one pill [17]. However, *in vivo* effects are uncertain, and lacking precise mechanisms of action, as even extracts of the same plant produced by different manufacturers do not necessarily have the same biological, or clinical effects, and may differ in composition [1,18]. In addition, extraction techniques may have an impact on the composition and biological activity of available products, *e.g.* based on extracts from the fruit of *Serenoa repens* [18]. Recently, Permixon®, hexane-extracted *Serenoa repens* (HESr), has been introduced to the updated European Association of Urology (EAU) guidelines on the management of non-neurogenic male LUTS [1]. The EAU's recommendation of HESr is the first guideline recommendation of any phytotherapeutic agent and based on two systematic reviews focused on data from fifteen randomized controlled trials (RCT) [19,20]. With less side effects and improvement of LUTS similar to that of α_1-blocker tamsulosin and short-term use of 5-ARI finasteride, HESr is the first phytotherapeutic agent being recommended by EAU guidelines [1,21]. Unlike ethanolic extracts of *Serenoa repens*, which are available as well but are not superior to placebo, HESr improved male LUTS with a degree similar to α_1-blockers [19,20]. Various studies have claimed mechanistic potential of *Serenoa repens* extracts *in vitro*, ranging from frequently described 5-AR inhibition to anti-inflammatory and anti-proliferative effects, and even suggested non-competitive α_1-adrenoceptor antagonism [22–25], or relaxing effects of chemical fractions of non-hexane-extracted *Serenoa repens* preparations on smooth contractions of non-human prostate tissues [26]. However, data are limited to non-urological, or non-human tissues [25,26], or were not reproducible *in vivo* [27], and improvement of male LUTS *in vivo* seems limited to HESr preparations [19,20] Thus, and to the best of our knowledge, there is no study able to explain the positive findings for HESr in clinical trials, as effects of Permixon® on human prostate and bladder smooth muscle contractility and prostatic cell growth have never been assessed. Therefore, we examined the effects of HESr (Permixon®) on human prostate and detrusor contractility, and on cellular functions of human prostate stromal cells.

2. Materials and methods

2.1. Materials, drugs and nomenclature

Permixon® (160 mg capsules) was purchased from Pierre Fabre Pharma AG (Allschwil, Switzerland). Permixon® 160 mg capsules contain free fatty acids, 80.7 % (mainly lauric, oleic, myristic and palmitic acids); glycerides, 6.8 %; methyl and ethyl esters, 2.5 %; unsaponified matter, 2.27 %; long-chain esters, 1.36 % [18]. N-Hexane (≥95 % purity, C_6H_{14}) was obtained from Carl Roth GmbH (Karlsruhe, Germany). Noradrenaline (4-[(1*R*)-2-amino-1-hydroxyethyl]-1,2-benzenediol), phenylephrine ((*R*)-3-[-1-hydroxy-2-(methylamino)ethyl] phenol), methoxamine (α-(1-aminoethyl)-2,5-dimeth-oxy-benzyl alcohol) are agonists for α_1-adrenoceptors. Carbachol (syn. carbamoylcholine; 2-hydroxyethyl)-trimethylammonium-chlorid-carbamat)) and methacholine (acetyl-β-methylcholin) are muscarinic acetylcholine receptor agonists [28]. U46619 ((Z)-7-[(1-*S*,4*R*,5*R*,6*S*)-5-[(E,3*S*)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid) is an analog of thromboxane A_2 and frequently used as an agonist for thromboxane receptors. Endothelin-1 is a 21-amino acid peptide with high affinity to the endothelin A (ET_A) and B (ET_B) receptors. Aqueous stock solutions of noradrenaline, phenylephrine, methoxamine, methacholine, and carbachol were freshly prepared before each experiment. Stock solutions of U46619 were prepared in ethanol and stock solutions of endothelin-1 in water, and both stored at −80 °C until use. Noradrenaline, phenylephrine, methoxamine, methacholine, and carbachol were obtained from Sigma (Munich, Germany), and U46619 and endothelin-1 from Enzo Life Sciences (Lörrach, Germany).

2.2. Preparation of Permixon®

In vivo, Permixon® 160 mg capsules are administered twice daily (once in the morning, and once in the evening), totaling to 320 mg/day [18,21]. For *in vitro studies*, Permixon® was previously used at a concentration of 10 μg/ml by Bayne and colleagues, which was described as a predicted physiological concentration, assuming distribution in total body fluid achievable using the recommended therapeutic dosage [24]. We therefore dissolved the contents of one capsule of Permixon® 160 mg with 10 ml n-hexane to a concentration of 16 mg/ml hexane-extracted Permixon® stock solution. For this purpose, one capsule of Permixon® 160 mg was opened and the contents were resuspended in 10 ml n-hexane, followed by incubation in a water bath (37 °C) for 2 h. Afterwards, the tube was centrifuged at 500 G for 3 min, and the

supernatant was aliquoted to 1 ml working solutions with a concentration of 16 mg/ml Permixon®. From these working solutions, varying amounts were applied to examine concentration-dependent effects. For example, 3 µl stock solution was taken, amounting to a total of 0.048 mg Permixon® and applied to an organ bath chamber containing 10 ml Krebs-Henseleit solution, resulting in a final concentration of 0.0048 mg/ml (*i.e.* 4.8 µg/ml). This dilution process was repeated with 10 and 30 µl of stock solution. The same procedure was repeated for all cell culture experiments, for example 1 µl stock solution, amounting to a total of 0.016 mg Permixon® was applied to 10 ml FCS-free medium to a final concentration of 0.0016 mg/ml (*i.e.* 1.6 µg/ml). This dilution process was repeated with 3, 10 and 30 µl of stock solution. Even though hexane volatilizes immediately in a heated organ bath or incubator [29], and therefore will most certainly not affect tissues or cells, the highest amount of hexane was used for hexane-treated controls (*i.e.* 30 µl diluted in 10 ml FCS-free medium) to eliminate any bias through hexane-associated effects.

2.3. Organ bath

For organ bath experiments, human prostate and detrusor tissues were obtained from patients who underwent radical prostatectomy for prostate cancer ($n = 103$), or radical cystectomy for bladder cancer ($n = 29$) at our tertiary referral center. While prostate tissues were obtained from male patients only, detrusor tissues were obtained from female and male patients. Our research was carried out in accordance with the Declaration of Helsinki of the World Medical Association and has been approved by the ethics committee of Ludwig-Maximilians University, Munich, Germany. Informed consent was obtained from all patients. All samples and data were collected and analyzed anonymously. Tissue sampling, and subsequent tension measurements were performed as described previously for prostate, and detrusor specimens, respectively [30,31]. In addition to construction of concentration response curves, EC_{50} values for contractile agonists, and Ef_{50} values for frequency-induced contractions (f), and E_{max} values were calculated by curve fitting. Curve fitting was performed separately for each single experiment, using GraphPad Prism 9.3.0 (GraphPad Software Inc., San Diego, CA, USA), and values were analyzed as described below. E_{max} values, EC_{50} values for contractile agonists, and frequencies (f) inducing 50 % of the maximum EFS-induced contraction (Ef_{50}) were calculated for each single experiment. As presentation of single values in scatter plots was intended, curve fitting was performed separately for each single experiment, resulting in separate values for each independent experiment. Frequency response curves and concentration response curves were fitted without predefined constraints for bottom, top, or EC_{50} values, by ordinary fit, without weighting, and without choosing automatic outlier elimination by non-linear regression. Resulting values were checked for plausibility and settings were adapted as follows if error messages occurred, as recommended in the "GraphPad Curve Fitting Guide" (GraphPad Software Inc., San Diego, CA, USA).

2.4. Cell culture

Cell culture experiments were performed with an immortalized cell line obtained from nonmalignant human prostate stroma (WPMY-1) as previously described [30]. Cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), and kept in RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10 % fetal calf serum (FCS) and 1 % penicillin/streptomycin at 37 °C with 5 % CO_2. Before addition of Permixon® or hexane, the medium was changed to FCS-free medium. Medium was further changed every day until cells were confluent. After cell counting and determination of the proportionate volume required for further experiments, cells were transferred to culture vessels for respective experiments.

2.5. Cell proliferation assay

Proliferation of WPMY-1 cells was assessed by "EdU-Click 555" cell proliferation assay (Baseclick, Tutzing, Germany), as previously described [30,31]. Confluent cells were treated with Permixon® or hexane, and grown for 24, 48 or 72 h. The number of proliferating cells (*i.e.* EdU-stained cells) was calculated as percentage of all cells individually for each sample.

2.6. Plate colony assay

Colony formation of WPMY-1 cells was assessed as previously described [30,31]. Confluent cells were either exposed to Permixon® or to hexane for 168 h, and the number of cell colonies was counted individually for each sample.

2.7. Cell viability assay

The effect of Permixon® on cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich, St. Louis, MO, USA), as previously described [30,31]. Confluent cells were exposed to Permixon® or hexane for 24, 48 and 72 h, and absorbance (optical density, OD) in each well was measured at 450 nm.

2.8. Flow cytometry analysis for apoptosis and cell death

A flow cytometry-based annexin V allophycocyanin (APC) and 7-aminoactinomycin D (7-AAD) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to detect cells in apoptosis (annexin V-positive, 7-AAD-negative) and dead cells (annexin V-positive, 7-AAD-positive) as previously described [30,31]. After addition of Permixon® or hexane, cells were incubated for 72 h and counted by flow cytometry.

2.9. Phalloidin staining

For fluorescence staining with phalloidin, cells were grown on Lab-Tek Chamber slides (Thermo Fisher, Waltham, MA, USA) with Permixon® or hexane, and stained with fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma-Aldrich, Munich, Germany) and analyzed as previously described [30,31].

2.10. Data and statistical analysis

Data in frequency and concentration response curves are presented as means ± standard deviation (SD), together with the indicated number (n) of independent experiments. E_{max}, pEC_{50}, Ef_{50}, IC_{50} values and data from cell culture are presented as single values from each independent experiment, together with means in scatter plots, where related samples from one experiment are indicated by corresponding symbols. Effect sizes become obvious from frequency and concentration response curves and from scatter plots, and are simplified summarized in the text. Statistical analyses were performed using GraphPad Prism Version 9.3.0 (GraphPad Software Inc., San Diego, CA, USA). Comparison of whole frequency/concentration response curves was performed by two-way analysis of variance (ANOVA). E_{max}, pEC_{50}, and Ef_{50} values were calculated by curve-fitting, and compared by a paired Student's *t*-test. Curve fitting was performed using GraphPad Prism 9.3.0 and limited to organ bath experiments, and to calculation of IC_{50} values in those series of cell culture experiments, where maximum effects were obviously attained in the applied concentration range. The present study and analyses show an exploratory design and were not designed to test a pre-specified statistical null hypothesis, for previously described reasons [32]. Accordingly, *p* values reported here need to be considered as descriptive, but not as hypothesis-testing. Minimum number of experiments and group sizes for each series were pre-planned as $n = 5$/group, to allow statistical analyses. Data were analyzed, after five or more

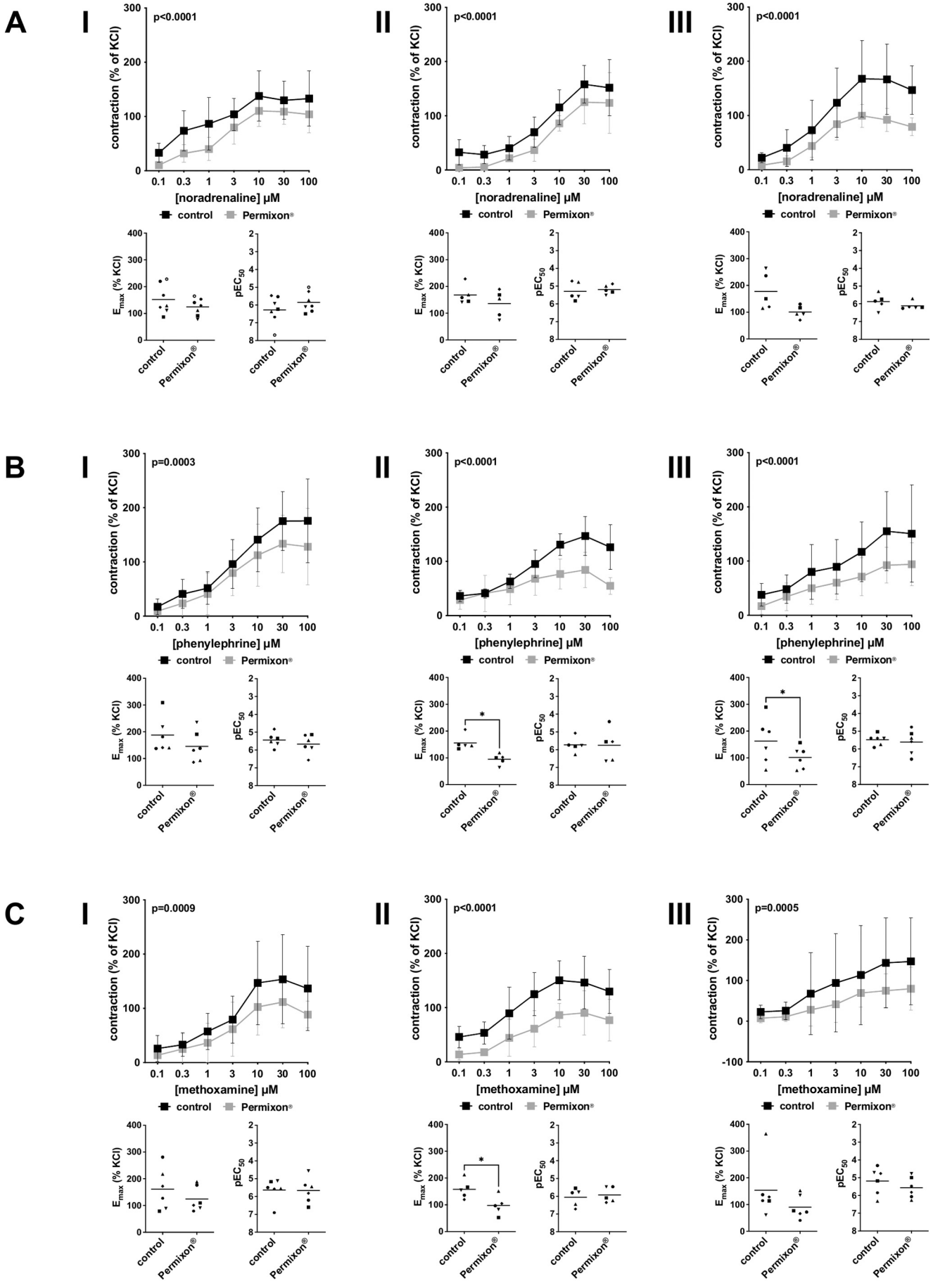

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Fig. 1. Effects of Permixon® on adrenergic contractions of human prostate tissues. Contractions were induced by the α_1-adrenoceptor agonists noradrenaline (A), phenylephrine (B), and methoxamine (C), after addition Permixon® extract at concentrations of 4.8 μl/ml (I), 16 μl/ml (II), and 48 μl/ml (III) (left to right), or hexane for controls. To eliminate heterogeneities due to individual variations, different degree of BPH, varying smooth muscle content or other confounders, tensions have been expressed as percentage of contraction by high molar KCl, being assessed before application of Permixon or hexane. Each experiment used strips from different patients ($n = 51$), and data are graphed as means ± SD from $n \geq 5$ different patients per individual series. Tissue from each patient was allocated to the control and drug group examined in the same experiment, resulting in paired groups.

experiments were performed for a given series. If these initial results were inconclusive, *i.e.* pointed to a possible drug effect but without p values <0.05, series were continued and analyzed again. This procedure was possible due to the explorative character and as long as it is reported in detail [32], and flexible group sizes have been recommended for data being characterized by large variations [33]. However, interim analyses were limited to frequency and concentration response curves and did not include E_{max} values, which were calculated only after completion of a series. No data or experiments were excluded from analyses.

3. Results

3.1. Effects of Permixon® on contractions of human prostate tissues

The α_1-adrenergic agonists noradrenaline, methoxamine and phenylephrine, and the non-adrenergic agonists U46619 and endothelin-1 induced concentration-dependent contractions of human prostate tissues, which were examined after addition of Permixon® in different concentrations (4.8 μg/ml, 16 μg/ml, 48 μg/ml) and of equivalent amounts of hexane in controls. Neurogenic contractions were induced frequence-dependently by EFS. Contractions induced by noradrenaline, methoxamine and phenylephrine were inhibited partly by Permixon® in each of the three examined dilutions (Fig. 1A–C). The degree of inhibition was similar with all three α_1-adrenergic agonists, with inhibitions ranging mostly around one-third or higher (Fig. 1A–C). Contractions induced by U46619 were inhibited as well by Permixon®, again by each examined dilution of Permixon® and around one-third or up to 50 % (Fig. 2A). The degree of inhibition was obviously highest at 48 μg/ml Permixon® extract (Fig. 2A). Endothelin-1-induced contractions were not changed by Permixon® (Fig. 2B). EFS-induced contractions were inhibited with 16 μg/ml and 48 μg/ml Permixon® extract, mostly around 60 %, but not or to neglectable extent using 4.8 μg/ml Permixon® extract (Fig. 2C). Significant reduction of E_{max} values for phenylephrine-, methoxamine- and U46619-induced contractions by Permixon® extract occurred with dilutions of 16 μg/ml and 48 μg/ml, while an elevation of EC_{50} values for U46619 occurred with 48 μg/ml Permixon® extract (Figs. 1, 2A).

3.2. Effects of Permixon® on contractions of human detrusor tissues

The cholinergic agonists carbachol and methacholine, and the non-cholinergic agonists U46619 and endothelin-1 induced concentration-dependent contractions of human detrusor tissues, which were examined after addition of Permixon® extract (16 μg/ml) and of equivalent amounts of hexane in controls. Neurogenic contractions were induced frequence-dependently by EFS. Contractions induced by methacholine were inhibited by Permixon® around 50 %, while carbachol-induced contractions remained unchanged (Fig. 3A, B). U46619-induced contractions were inhibited slightly, *i.e.* <30 %, while endothelin-1-induced contractions were not changed (Fig. 3C, D). EFS-induced contractions were inhibited to neglectable extent at most frequencies, but around 25 % at 16 Hz (Fig. 3E). E_{max} values were significantly reduced for methacholine-induced contractions (Fig. 3B), and EC_{50} values were increased for U46619 (Fig. 3C).

3.3. Effects of Permixon® on proliferation of WPMY-1 cells

WPMY-1 cells were exposed to different amounts of Permixon® extract (1.6–48 μg/ml) and to equivalent amounts of hexane in controls. Permixon® reduced the proliferation rate in WPMY-1 cells in a concentration-dependent and time-dependent manner (Fig. 4A–C). Inhibition of proliferation was largest with the highest concentration of Permixon® extract, consistently amounting to 25 % inhibition for each incubation period.

3.4. Effects of Permixon® on colony formation of WPMY-1 cells

Colony formation of WPMY-1 cells was reduced by Permixon® (Fig. 5A). The decline in colony formation was concentration-dependent and obviously reached a maximum at the highest applied concentration of 48 μg/ml Permixon® extract, amounting to a reduction of about 50 %. The IC_{50} for inhibition of colony formation amounted to 32 μg/ml [3 to 60].

3.5. Effects of Permixon® on viability of WPMY-1 cells

Permixon® (1.6–48 μg/ml) reduced the viability of WPMY-1 cells in CCK-8 assays (Fig. 5B–D). Reduction of viability was concentration- and time-dependent, approaching to virtually completely reduced viability after 48 and 72 h with the highest two concentrations of Permixon® extract (Fig. 5C, D). Curve fitting revealed an IC_{50} of Permixon® extract for inhibition of viability of 38 μg/ml [20 to 57], 7.9 μg/ml [7.3 to 8.5], and 6.5 μg/ml [6.3 to 6.6] for 24, 48 and 72 h, respectively.

3.6. Effects of Permixon® on apoptosis and cell death of WPMY-1 cells

Effects of Permixon® (1.6–48 μg/ml μl) on apoptosis and cell death in WPMY-1 cells were assessed by flow cytometry analysis for annexin V and 7-AAD, where annexin V-positive/7-AAD-negative cells represent cells in apoptosis (Fig. 6A), and annexin V-positive/7-AAD-positive cells represent dead cells (Fig. 6B). Even at the highest concentration of 48 μg/ml, Permixon® did not increase the relative numbers of cells in apoptosis or of dead cells compared to hexane-treated controls (Fig. 6C).

3.7. Effects of Permixon® on actin organization of WPMY-1 cells

Phalloidin-stained actin filaments in hexane-treated control cells were arranged to bundles of long and thin protrusions, and elongations from adjacent cells were overlapping each other (Fig. 7). Permixon® (1.6–48 μl) caused concentration-dependent degradation of actin filaments after incubation for 24, 48 and 72 h, resulting in a rounded cell shape without any protrusions (Fig. 7A–C). At the highest concentration, Permixon® (48 μg/ml) caused obvious and robust regression of phalloidin-stained areas, amounting up to 65 %. The IC_{50} for breakdown of polymerized actin by Permixon® extract amounted to 38 μg/ml [21 to 55], 6 μg/ml [3 to 9] and 13 μg/ml [10 to 16] at 24, 48, and 72 h, respectively.

4. Discussion

With the 2021 update of the EAU's guidelines on management of non-neurogenic male LUTS, a recommendation for the phytotherapeutic agent HESr has been made, which represents the first ever recommendation for a plant extract by these urologic guidelines [1]. Previous clinical trials suggested improvements of male LUTS by HESr, which are superior to placebo and comparable to α_1-blockers, whereas underlying

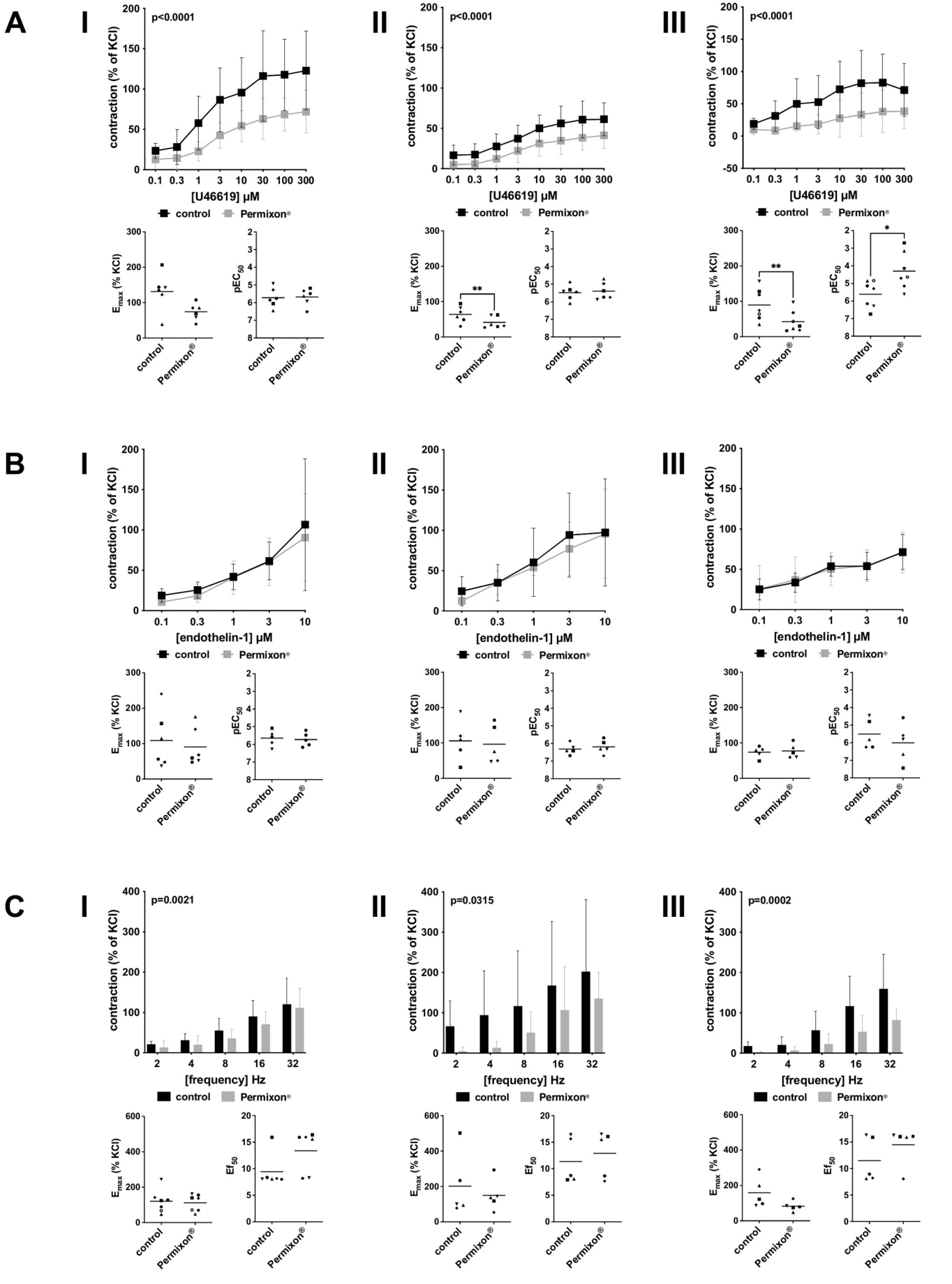

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Fig. 2. Effects of Permixon® on non-adrenergic and neurogenic contractions of human prostate tissues. Non-adrenergic contractions were induced by U46619 (A), and endothelin-1 (B), and neurogenic contractions were induced by EFS (C), after addition of Permixon® extract at concentrations of 4.8 μl/ml (I), 16 μl/ml (II), and 48 μl/ml (III) (left to right), or hexane for controls. To eliminate heterogeneities due to individual variations, different degree of BPH, varying smooth muscle content or other confounders, tensions have been expressed as percentages of contraction by high molar KCl, being assessed before application of Permixon® or hexane. Each experiment used strips from different patients ($n = 52$), and data are graphed as means ± from $n \geq 5$ different patients per individual series. Tissue from each patient was allocated to the control and drug group examined in the same experiment, resulting in paired groups.

mechanisms have been rarely and incompletely addressed, and are unknown. Our current findings point to concentration-dependent inhibition of smooth muscle contractions in human prostate and detrusor tissues, and simultaneous inhibition of prostate stromal cell growth. These functions are critical for the development of male LUTS and represent important targets of standard-of-care medical therapy. For the first time in current literature, our findings fully allow to explain the improvements of male LUTS by HESr.

Smooth muscle contractility is one of the two main targets in LUTS pharmacotherapy, as exaggerated prostate smooth muscle contraction contributes to BOO and uncontrolled bladder smooth muscle contraction contributes to OAB [3,6]. It has become increasingly obvious that, in addition to α_1-adrenoceptors, other mediators are also involved as agonists in smooth muscle contraction [34]. At concentrations ranging from 1.6 to 48 μg/ml, we observed inhibition of adrenergic prostate smooth muscle contraction of 30 to 60 %, which increased with amounts of applied Permixon® extract. This complements previous findings using different *Serenoa repens* extracts, obtained by different extraction techniques, and solvents, which pointed to shared effects, *i.e.* non-competitive binding to α_1-adrenoceptors, while the potency and efficacy obviously strongly varied between extracts [23]. Even though the binding of different *Serenoa repens* extracts to α_1-adrenoceptors suggested a class effect, the varying potency may explain, why there only is evidence for efficacy *in vivo* from clinical trials for HESr, but not for other extraction methods, like ethanol-extracted *Serenoa repens fructus*. A subsequent *in vivo* study confirmed lacking α_1-adrenoceptor antagonism by *Serenoa repens* extracts and pointed to non-competitive binding to α_1-adrenoceptors as a class effect of *Serenoa repens* extracts again, although this study did not include Permixon® [27].

Surpassing possible α_1-adrenoceptor antagonism, we found that Permixon® also inhibited non-adrenergic contractions by the thromboxane-analog U46619, while tamsulosin has no effect on thromboxane-induced prostate smooth muscle contractions [34]. In fact, our findings with α_1-adrenergic agonists (where concentration response were not right-shifted, but E_{max} values were obviously decreased) and with non-adrenergic agonists (where contractions with U46619 and methacholine were partly inhibited) are in line with previous studies reporting lacking antagonism by *Serenoa repens* extracts [23,27]. Interestingly, we observed no effect of Permixon® on endothelin-induced contractions at any concentration, suggesting that the anticontractile effects on other mediators were not attributed to an unspecific inhibitory effect, *e.g.* due to cell death during organ bath experiments.

Neurogenic contractions by EFS were inhibited by Permixon® in both tissues, and in a concentration-dependent manner at least in prostate tissues. Neurogenic contractions of smooth muscle tissues by EFS are caused by stimulation of neuronal action potentials, subsequent release of endogenous neurotransmitters (noradrenaline in prostate tissues, acetylcholine in bladder tissues) and subsequent activation of postsynaptic α_1-adrenoceptors or M3 muscarinic receptors, respectively [35]. In prostate tissues, Permixon® inhibited EFS-induced contractions around 60 %, using the highest applied concentration of Permixon® extract. The extent of this inhibition is fully in the range of α_1-blockers, amounting to 48–76 % inhibition of EFS-induced contraction by tamsulosin or 47 % by silodosin, using human prostate tissues under similar experimental conditions in our previous studies [35–37]. In detrusor tissues, the extent of inhibition of EFS-induced contractions by Permixon® was obviously smaller as in prostate tissues, what also applies to U46619-induced contractions.

The susceptibility of cholinergic bladder smooth muscle contractions to Permixon® differed with the cholinergic agonist, as we observed strong inhibition of methacholine-induced contractions but no effect in carbachol-induced contractions. Detrusor smooth muscle mainly contains M2 and M3 subtypes of muscarinic receptors at a ratio of 3:1 [38]. M3 is coupled to Gq and mediates contraction of bladder smooth muscle, whereas M2 is presumably coupling to Gi and does possibly not mediate contraction [28,31,38]. Methacholine binds in an unspecific manner to all five muscarinic receptors (M1–5), whereas carbachol does not bind to M2 receptors [28]. However, and to what extent binding of methacholine to the M2 receptor may have had an effect, *i.e.* by increasing its affinity to M2 in the presence of Permixon®, or by altering the conformational state, remains speculative at this stage. As inhibitions of non-cholinergic contractions in detrusor tissues remained incomplete, it may be concluded that this phenomenon is not limited to muscarinic receptors but may apply to all contractile receptors in bladder smooth muscle.

While spasmolytic effects on noradrenaline-induced contractions of rat aorta has been suggested, our data point to anti-contractile activity of HESr, using an array of contractile agonists on human prostate and bladder tissues [25]. The effects of Permixon® on smooth muscle contraction may be either due to non-competitive receptor antagonism, as previously suggested [23], or by any other kind of inhibition of downstream post-receptor signaling. However, identification of active compound(s) and of targets was not the aim of our study, and we did not compare different extracts of *Serenoa repens*. It appears both possible, that either one single compound in the extract accounts for all effects seen in organ bath experiments and cell culture, but also that several active compounds account for the different observed effects. Even though Permixon® caused obvious breakdowns of actin polymerization in our cell culture experiments, this cannot explain inhibitions of smooth muscle contraction in our organ bath experiments, considering that endothelin-1-induced contractions remained unaffected by Permixon®. Thus, as correct organization and adequate polymerization of actin filaments are prerequisites for any type of smooth muscle contraction, inhibition of endothelin-1-induced contractions should be seen as well, if actin breakdown occurred in organ bath experiments. In fact, Permixon® was applied for 30 min the organ bath, but for 24–72 h in cell culture. *In vivo*, inhibition of endothelin-1-induced contractions may be possible, where the exposure is long and actin breakdown may consequently occur.

While a large meta-analysis of 30 RCTs, including 5222 men, follow-ups ranging from 4 to 60 weeks, and ethanol- and hexane-extracted *Serenoa repens* demonstrated no benefit of *Serenoa repens* extracts in comparison to placebo for the reduction of LUTS, the EAU guideline recommendation is mainly based on the results of two systematic reviews of 12 and 15 RCTs, respectively, including only HESr preparations [19,20,39]. Compared with placebo, HESr was associated with reduction of nocturia (*i.e.* fewer voids/night), and an additional mean increase in Q_{max} of 2.75 ml/s. Most interestingly, HESr showed similar improvements in IPSS and a comparable increase in Q_{max} as the α_1-blocker tamsulosin. Even between HESr and 5-ARIs, efficacy was similar after six months of treatment, respectively [20]. In general, a decrease in IPSS of $\geq$3 is perceptible to the patient and must be achieved for new medications to be considered effective [40]. Interestingly, the analysis of all available published data for HESr showed a mean significant improvement in IPSS from baseline of 5.73 points, marking the threshold of three

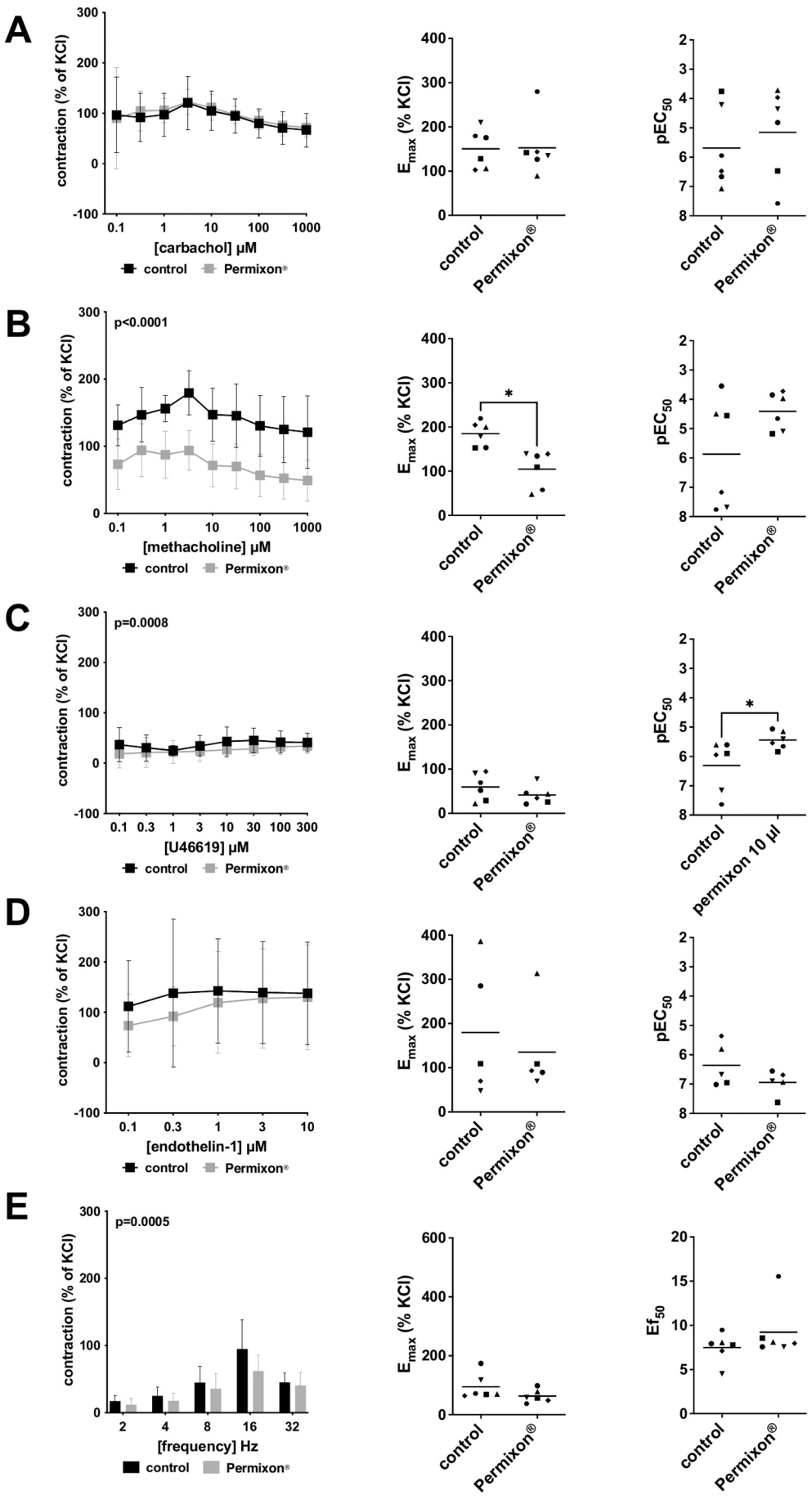

Fig. 3. Effects of Permixon on cholinergic and non-cholinergic, and EFS-induced neurogenic contractions of female and male human detrusor tissues. Cholinergic contractions were induced by the m-cholin receptor agonists carbachol (A), and methacholine (B), while non-cholinergic contractions were induced by thromboxane A_2 analog U46619 (C) and endothelin-1 (D), and neurogenic contraction was induced by EFS (E), after addition of Permixon (16 µg/ml) or hexane for controls. To eliminate heterogeneities due to individual variations, or varying smooth muscle content or other heterogeneities, tensions have been expressed as percentages of contraction by high molar KCl, being assessed before application of Permixon or hexane. Data are means ± SD from series with tissues from $n = 6$ male patients for carbachol (A), $n = 4$ female and $n = 2$ male patients for methacholine (B), $n = 4$ other female and $n = 2$ other male patients for U46619 (C), $n = 5$ male patients for endothelin-1 (D), and $n = 6$ other male patients for EFS (E). Tissue from each patient was allocated to the control and drug group examined in the same experiment, resulting in paired groups.

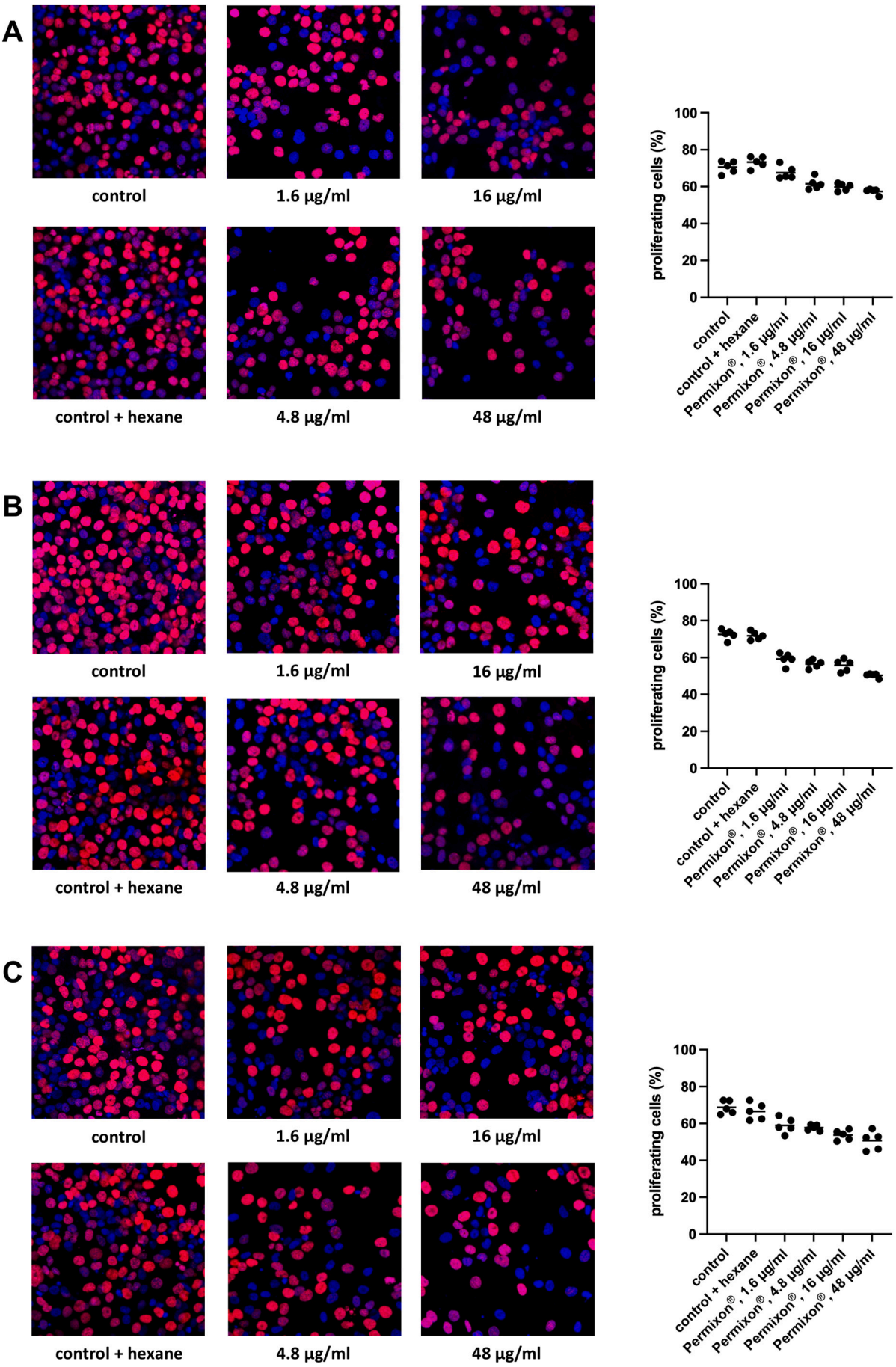

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Fig. 4. Inhibition of prostate stromal cell proliferation by Permixon using concentrations of 1.6–48 μg/ml. Shown are the percentages of proliferating cells (single values from each experiment together with means) for each concentration after 24 h (A), 48 h (B), and 72 h (C), using cell cultures from $n = 5$ independent experiments for each concentration and time. The cells were either allocated to a control (no solvent), or control (hexane) or Permixon® groups, and incubated for 24, 48, and 72 h, respectively. Proliferating cells were detected by EdU staining and counterstaining of all nuclei with DAPI, resulting in blue-colored nuclei for non-proliferating cells and red nuclei for proliferating cells. Shown are exemplary images of cell proliferation after 24, 48, and 72 h (left), and quantification of all experiments (right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

points [20]. For comparison, representative values for α_1-blockers ranged around increases in Q_{max} around 0.7–2.5 ml/s and decreases in IPSS 3.8–6.6 points, in seminal clinical trials [41,42].

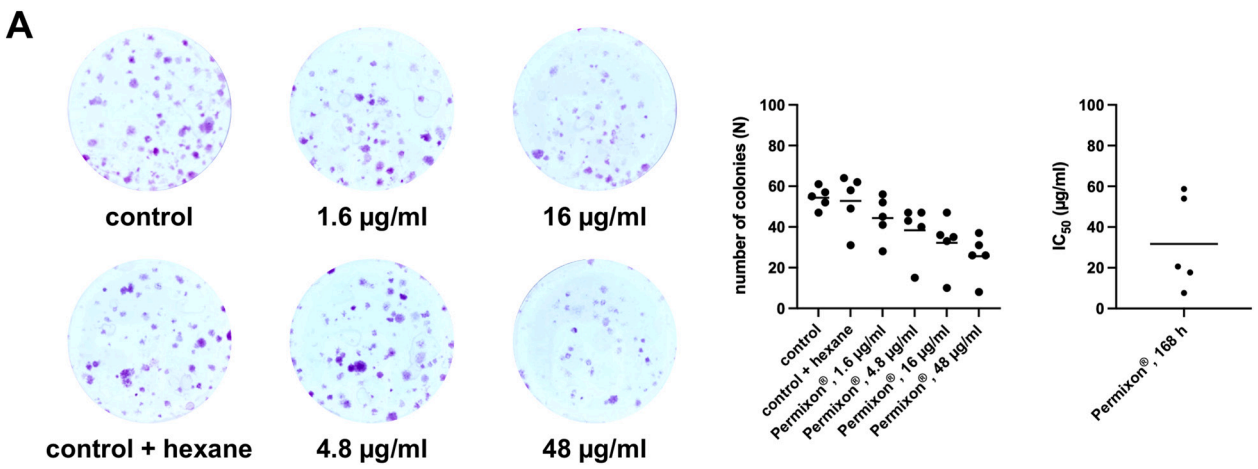
With respect to safety and tolerability in an elderly patient population, the data from the systematic reviews showed that HESr had a favorable safety profile, with gastrointestinal disorders as the most frequent adverse events (3.8 %) [20]. The most recent study on comparing tolerability and efficacy of HESr with tamsulosin reported only 2.1 % adverse events (AEs) in patients receiving HESr, compared to 14.7 % in the tamsulosin group [21]. Overall, clinical trials suggest high efficacy, combined with excellent tolerability for HESr, such as Permixon®. While an abundance of clinical trials has emerged since *in vitro* effects of Permixon® became known in the late 1990s and early 2000s, the exact mechanisms of action still remain obscure.

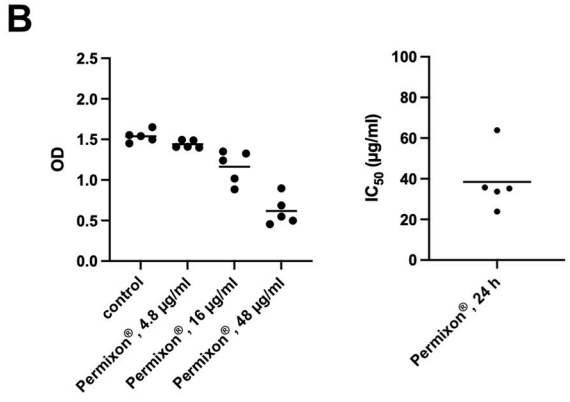
Previous studies addressing HESr effects on prostate cells or tissues *in vitro* were few, and mostly limited to examine apoptosis or cell death. While Bayne et al. could not determine whether Permixon® had any effect on the apoptotic profile of the human prostate, several of the morphological changes observed in their coculture experiments, including polarization of the nucleus and condensation of chromatin following treatment with Permixon®, suggest that Permixon® may induce apoptosis in both the epithelial and stromal cells [24]. In our study, we used an immortalized prostate stromal cell line (WPMY-1) to assess effects of Permixon® on apoptosis and cell death using flow cytometry. Regardless of the concentration used, Permixon® did not induce apoptosis or cell death. Even at the 4.8-fold recommended *in vitro* dose (30 μl, *i.e.* 48 μg/ml), cells did not show signs of increased apoptosis or cell death, compared to hexane-treated controls. However, Vacherot et al. compared the rate of cell proliferation and apoptosis in normal prostate tissues obtained from organ donors and from patients with BPH treated or untreated with Permixon® for 3 months [43]. The ratio of epithelial-to-stromal cells was found to be significantly higher in normal prostate tissue than in BPH. As BPH is a true hyperplastic process, proliferation exceeded apoptosis in their BPH tissue. Study subjects treated with Permixon® for 3 months, showed reversed apoptosis/proliferation ratio, with significantly higher apoptotic index than the proliferative index in the epithelium and stroma, increasing by 5.5 and 8.8-fold, respectively. Additionally, treatment with Permixon® decreased the proliferative index compared to untreated BPH cases by up to 7.7-fold [43]. While we cannot corroborate the data on pro-apoptotic features of Permixon®, we observed significant, albeit limited, reduction of prostate stromal cell proliferation of up to 30 % for the highest concentration of Permixon® after incubation for 48 h.

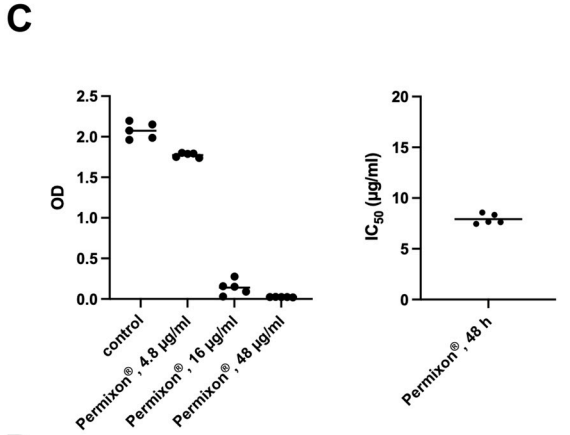
Paubert-Braquet et al. investigated the effect of Permixon® on bFGF- and EGF-stimulated proliferation of prostate cells *in vitro* in human biopsy material obtained from enucleated prostate adenomatous tissue [44]. While Permixon® did not inhibit basal prostate cell proliferation, it significantly inhibited the b-FGF- and EGF-induced cell proliferation of human prostate explants in a dose-dependent manner. The inhibitory effects for b-FGF were always observed at the dose of 30 μg/ml, inhibition was noted at lower doses (1 and 10 μg/ml) only in some prostate samples. The inhibitory effect of Permixon® was less pronounced with EGF since some prostate specimens did not respond to treatment. Nevertheless, Permixon® also significantly affected EGF-induced cell proliferation at 30 μg/ml and in some cases also influenced proliferation at lower doses [44]. This is in accordance with our observations for prostate stromal cell proliferation. Inhibition was weak for lower concentrations of Permixon®, but increasingly obvious for 10 and 30 μl, corresponding to a final concentration of 16 and 48 μg/ml, respectively. However, inhibition of prostate growth in cell colony assay was more pronounced and amounted to over 50 % for the highest two concentrations used (*i.e.* 10 and 48 μg/ml Permixon®), with an IC_{50} value around 32 μg/ml. While viability of WPMY-1 cells was completely suppressed after treatment with 16 and 48 μg/ml Permixon®, resulting in an IC_{50} value of 6.5 μg/ml, this was not due to cytotoxic effects. Together this may reflect a shared susceptibility of proliferation and growth of prostate stromal cells to Permixon®, which differed with conditions applied in EdU and colony formation assays. The ability of WPMY-1 cells to form adherent cell colonies is due to their karyotypically altered state. Together, significant inhibition of collective cell growth by Permixon® in cell colony assay may further support their inhibitory effect on prostate growth [44,45]. To date, however, inhibition of prostate cell growth is limited to *in vitro* studies, and clinical trials have yet to prove such an effect *in vivo* [1,20,21].

Although the specific mechanisms of action of *Serenoa repens* fruit extracts have not yet been completely understood, it has been reported that the therapeutic agents could exert an inhibitory activity towards 5-AR, in addition to pro-apoptotic, anti-estrogenic and anti-inflammatory properties [22,46]. The presumed bioactive components of *Serenoa repens* include fatty acids and phytosterols. *Serenoa repens fructus* extracts predominantly consist of fatty acids and are a rich source of the saturated, medium-chain fatty acids laurate and myristate [47,48]. Some free fatty acids, such as lauric and myristic acid may even prevent testosterone-induced BPH in rats [49], or inhibit phenylephrine-induced vas deferens contractions [50], while palmitic acid may inhibit prostate cancer cell proliferation [51]. Chua and colleagues even proposed that the active fatty acids present in the liposterolic extract of *Serenoa repens fructus* may have a substantial smooth muscle relaxant effect [26]. However, in using a concentration of 1 mg/ml liposterolic *Serenoa repens* extract, it seems unlikely that the recommended daily dose of this extract (320 mg) would translate to an *in vivo* effect. In addition, these results are contrasted by sympathomimetic properties, found for ethanol-extracted *Serenoa repens* by members of the same group [52,53]. In summary, data are limited to non-human tissues (*i.e.* rodents), and prostate cancer cells, and may not be extrapolated to WPMY-1 cells, or human detrusor and prostate tissues. In addition, inhibitory effect on 5-AR were mostly performed *in vitro* and their relevance *in vivo* needs to be validated.

While phytosterols are relatively minor constituents of *Serenoa repens* fruit extracts [47], other studies suggest that phytosterols (β-sitosterol, campesterol, stigmasterol), inhibit 5-AR, prostate cancer cell growth, and BPH symptoms [54–58]. However, these phytosterols are not unique to *Serenoa repens* extracts [59]. Nevertheless, a combination of fatty acids, phytosterols, and other bioactive components may be responsible for beneficial effects reported from *Serenoa repens* supplements. Regarding bioavailability of mechanistically relevant molecules, plasma concentrations of an unspecified component of HESr were measured in healthy young male volunteers ($n = 12$), receiving a single







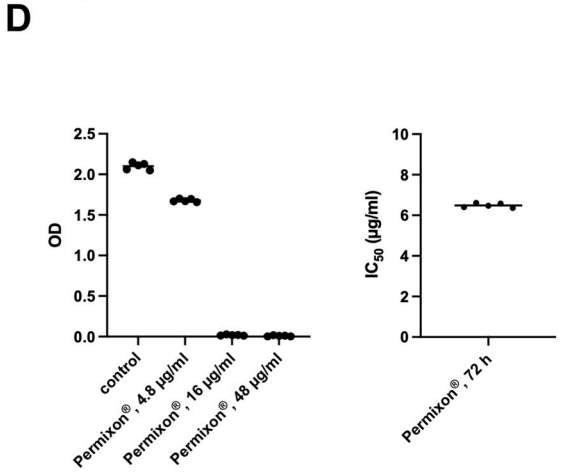


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Fig. 5. Effects of Permixon® on cell colony formation and viability of prostate stromal cells. Shown are the absolute numbers of colonies (N) after 168 h (single values with means) from a series using cell cultures from $n = 5$ independent experiments (A). The cells were either allocated to a control (no solvent), control (hexane), or Permixon® (1.6–48 μg/ml) group and incubated for 168 h. Shown are exemplary images of colony formation after 168 h (left), and quantification of all experiments (right) with corresponding IC_{50} values. To assess viability, a CCK-8 assay was used, and values are graphed as optical density (OD) from $n = 5$ individual experiments. The cells were either allocated to a control (no solvent), control (hexane) or Permixon® (4.8–48 μg/ml) group and incubated for 24, 48, and 72 h (B–C, respectively). Shown are the quantifications of all experiments as optical density (OD; mean ± SD) (left) with corresponding IC_{50} values from all five experiments (right).

oral dose of 320 mg HESr in fasting state [60,61]. Post-dose, the maximum plasma concentration (C_{max}) of 2.6 mg/L was achieved at 1.5 h, with mean area under the concentration-time curve of 8.2 mg/L per hour, and an elimination half-life of 1.9 h [61]. In rats, tissue distribution of HESr was assessed with radiolabeled oleic acid, lauric acid, or β-sitosterol after oral administration, showing higher uptake of radioactivity in the prostate gland than in the bladder, or seminal vesicles, or more remote sites (*e.g.* liver), further complementing our findings [62]. However, due to the large individual variability of the different preparations of *Serenoa repens* fruit extracts (*e.g.* different extraction techniques, and individual composition by different distributors), the bioavailable concentration of these fatty acids, and other bioactive ingredients in the human prostate tissue remains unclear [18,47,63].

Our study poses several limitations. While some of these bioactive ingredients may have entered our preparation of Permixon® extract, we feel it is unlikely that the observed effects on such a wide variety of panels may be explained by the bioactive components, as they are also part of other *Serenoa repens* fruit extracts (*e.g.* ethanol), which have no proven or relevant effect *in vivo* [18,46]. Also, the content of bioactive ingredients varies between batches from the same preparation, making it difficult to standardize and compare the potency of different extracts, or even different batches [64,65]. Therefore, clinical benefits derived from different extracts will vary depending on the solvent used for extraction of *Serenoa repens fructus*. Furthermore, our data were generated *in vitro*, which may not necessarily be translated to effects *in vivo*. However, our study is the first to demonstrate inhibitory action of HESr on human prostate and bladder smooth muscle contraction. Our observations complement previous studies, suggesting non-competitive α_1-adrenoceptor binding [23,25], but simultaneously provide a novel explanation for the ubiquitously beneficial effects *in vivo* by demonstrating efficacy of Permixon® on a wide array of contractile agonists in smooth muscle-rich organs of the lower urinary tract. However, and even though previous studies have suggested a possible class effect [23], our conclusions are limited to the Permixon® preparation.

5. Conclusion

Together our findings suggest a basis for the ubiquitously positive findings of HESr (Permixon®) addressing improvement of LUTS in clinical trials. HESr may inhibit at least neurogenic, α_1-adrenergic and thromboxane-induced smooth muscle contraction in the prostate and, although to weaker extent, in the detrusor, which may explain symptom improvements by Permixon® in previous clinical trials. In parallel, Permixon® inhibited proliferation and growth of stromal cells, which may contribute to beneficial effects *in vivo* as well.

Funding

This work was supported by grants from LMU Munich's Medical Faculty Förderprogramm für Forschung und Lehre (FöFoLe Reg.-Nr. 1092), Ferdinand Eisenberger-Forschungsstipendium der DGU (grant TaA1/FE-21) and the by grants from Deutsche Forschungsgemeinschaft (grants HE 5825/9-1). The funding sources had no part in study design, in the collection, analysis or interpretation of data, in the writing of the report, or in the decision to submit the article for publication.

Ethics statement

Our research was carried out in accordance with the Declaration of Helsinki of the World Medical Association and has been approved by the ethics committee of Ludwig-Maximilians University, Munich, Germany (ref. no. 19-738). Informed consent was obtained from all patients. All samples and data were collected and analyzed anonymously.

Data availability statement

All datasets generated for this study and supporting the findings of this study are included in the manuscript. The raw data of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Alexander Tamalunas: Conceptualization, Methodology, Validation, Formal analysis, Funding acquisition, Supervision, Visualization, Writing – Original Draft.

Amin Wendt: Investigation, Data Curation, Formal Analysis.

Florian Springer: Investigation, Data Curation.

Victor Vigodski: Investigation, Data Curation.

Anna Ciotkowska: Investigation, Data Curation, Formal Analysis, Visualization.

Beata Rutz: Investigation, Data Curation, Formal Analysis, Visualization.

Ruxiao Wang: Investigation, Data Curation.

Ru Huang: Investigation, Data Curation.

Yuhan Liu: Investigation, Data Curation.

Heiko Schulz: Investigation, Data Curation, Resources.

Stephan Ledderose: Investigation, Data Curation, Resources.

Thomas Kolben: Supervision, Writing – Review & Editing.

Giuseppe Magistro: Supervision, Software, Writing – Review & Editing.

Christian G. Stief: Supervision, Funding acquisition, Writing – Review & Editing.

Martin Hennenberg: Conceptualization, Writing – Review & Editing, Funding acquisition, Resources, Formal analysis, Supervision, Project administration.

All authors corrected the draft. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

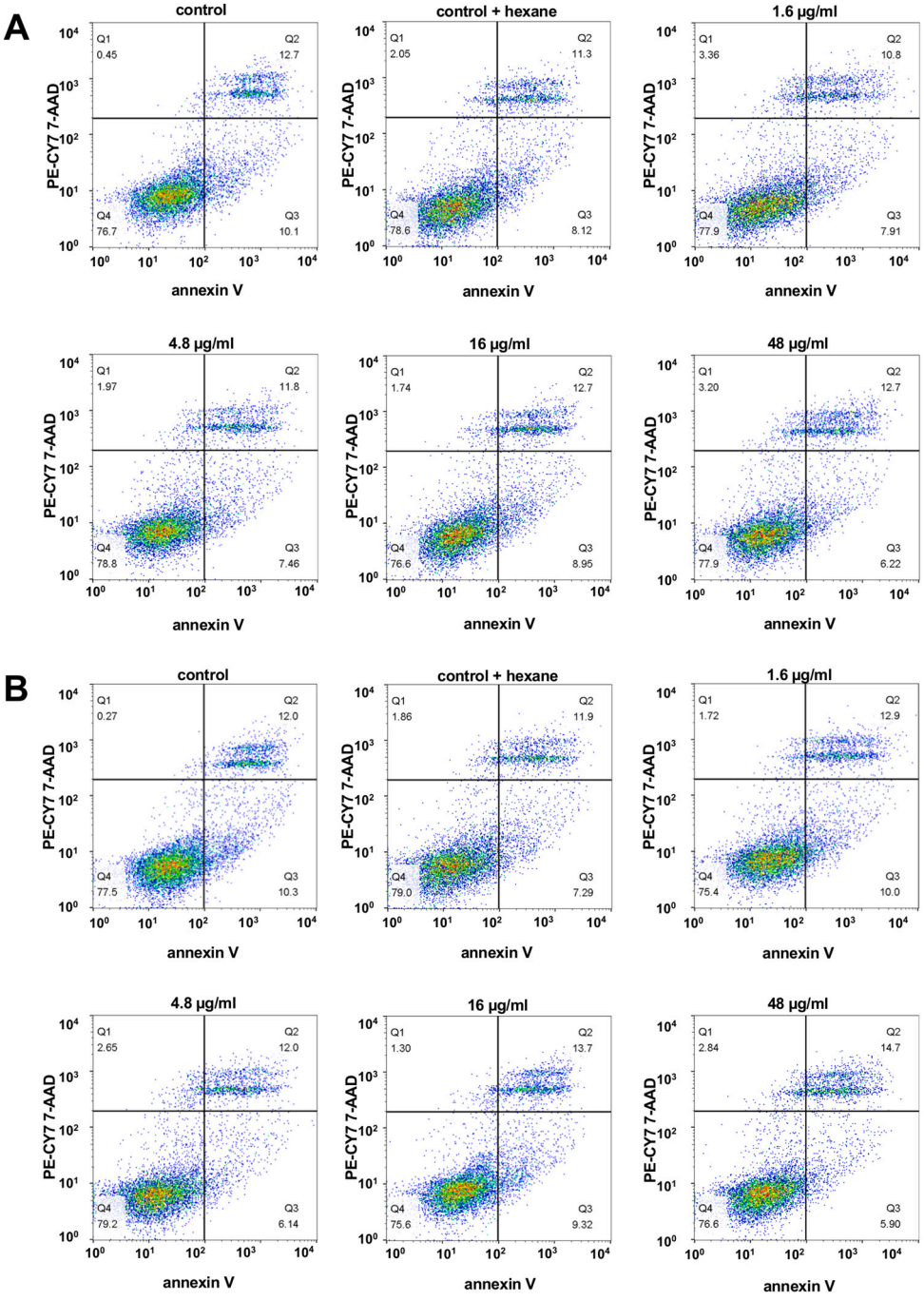

Fig. 6. Apoptosis (A) and cell death (B) in prostate stromal cells after stimulation with Permixon (1.6–48 µg/ml). Flow cytometry was performed, after cells were allocated to a control (no solvent), control (hexane), or Permixon® group, and treated for 72 h. Subsequently, the numbers of cells being in apoptosis (annexin V-positive, 7-AAD-negative), and of dead cells (resulting from apoptosis and/or necrosis; annexin V-positive, 7-AAD-positive) were assessed by flow cytometry. Shown are single values from each experiment together with means (percentage of cells in apoptosis, or of dead cells, referred to the number of all cells) (C), and representative single experiments (A, B) from a series of $n = 5$ independent experiments.

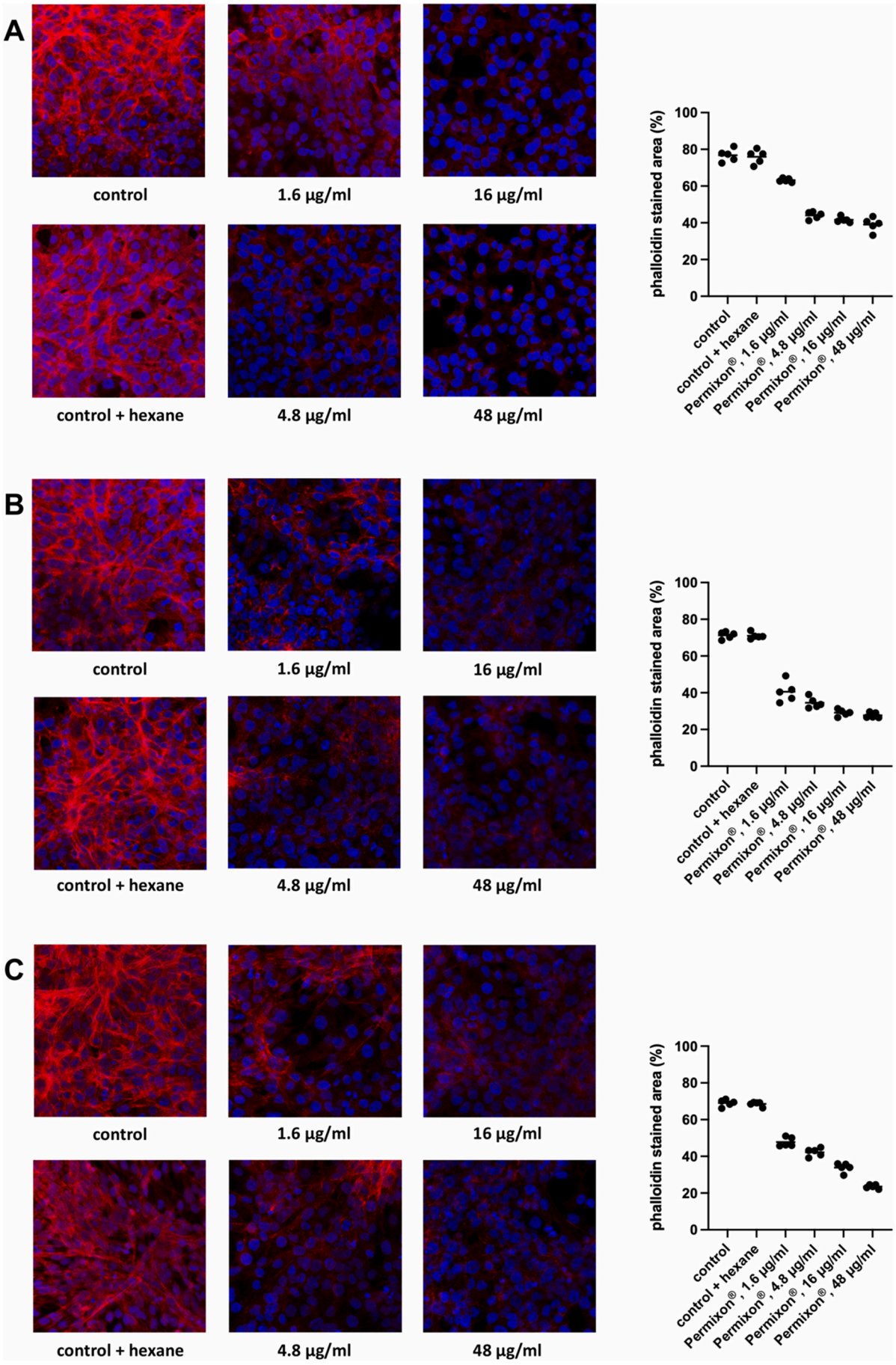

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Fig. 7. Inhibition of prostate stromal cell actin polymerization and organization by Permixon® using concentrations of 1.6–48 μg/ml. Shown are the percentages of phalloidin-stained areas (single values from all experiments together with means) for each concentration after 24 h (A), 48 h (B), and 72 h (C), using cell cultures from $n = 5$ independent experiments for each concentration and time. Cells were either allocated to a control (no solvent), or control (hexane) or inhibitor group, and incubated for 24, 48, and 72 h, respectively. Actin filaments were visualized by phalloidin staining, while the nuclei were visualized using DAPI staining. Shown are exemplary images of cell proliferation after 24, 48, and 72 h (left), and quantification of all experiments (right).

Declaration of competing interest

Thomas Kolben holds stock of Roche AG and a relative is employed at Roche AG. The other authors have no conflict of interest to declare.

Data availability

Data will be made available on request.

Acknowledgements

We thank Prof. Dr. Frederik Klauschen (Institute of Pathology, Ludwig-Maximilians University, Munich) and his coworkers for the asservation of tissue samples from prostates and bladders. We thank the team of “Metzgerei Brehm” (Planegg, Germany) for providing us pig kidneys and hearts. We thank Prof. Elfriede Noessner and her coworkers (Institute of Molecular Immunology, Helmholtz Center, Munich) and Dr. Maximilian Saller (Department of Orthopaedics and Trauma Surgery, Musculoskeletal University Center Munich (MUM), University Hospital, LMU Munich, Germany) for support with immunofluorescence microscopy.

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