Inhibition of human prostate stromal cell growth and smooth muscle contraction by thalidomide: A novel remedy in LUTS?

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Abstract

Background: Medical treatment in benign prostatic hyperplasia targets prostate size to prevent disease progression, complications, and surgery, and prostate smooth muscle tone for rapid relief of lower urinary tract symptoms. Combination therapies are still required to target both at once. However, current medications are insufficient, due to an unfavorable balance between side effects and efficacy. The limited efficacy of α1-blockers may be due to nonadrenergic mediators like endothelin-1 and thromboxane A2 (TXA2), which keep up prostate smooth muscle contraction even in the presence of α1-blockers. Consequently, future options with higher efficacy need to target α1-adrenergic and nonadrenergic contractions as well as stromal cell growth at once. Thalidomide has been approved as an oral medication for various diseases, including the treatment of prostate cancer. Therefore, we investigated the effect of thalidomide on cellular functions of prostate stromal cells and human prostate smooth muscle contraction.

Methods: Cytoskeletal organization was visualized by phalloidin staining, cell growth was assessed by 5-ethynyl-2-deoxyuridine assay, cell viability by cell counting kit-8, and apoptosis and cell death by flow cytometry in cultured prostate stromal cells (WPMY-1). Contractions of human prostate tissues from radical prostatectomy were studied in an organ bath, where they were induced by the α1-adrenoceptor agonists methoxamine, noradrenaline, phenylephrine, and the nonadrenergic agonists endothelin-1 and TXA2 analog U46619, or electric field stimulation (EFS).

Results: Thalidomide significantly reduced the proliferation of WPMY-1 cells, which was time- and concentration-dependent (10–300 µM). In parallel, organization of actin filaments collapsed after treatment with thalidomide. Thalidomide (30–100 µM) inhibited noradrenaline-, phenylephrine-, and methoxamine-induced contractions, as well as nonadrenergic contractions induced by endothelin-1 and U46619, and neurogenic contractions induced by EFS. No reduction in viability and no increases in apoptosis or in cell death were observed in WPMY-1 cells.
Conclusions: Thalidomide impairs the growth of human prostate stromal cells, without showing a decrease in cell viability. In parallel, thalidomide inhibits adrenergic, neurogenic, and nonadrenergic contractions. This may be explained by a breakdown of the actin cytoskeleton. In vivo, urodynamic effects of thalidomide appear possible and may even exceed those of α1-blockers or combination therapies.

KEYWORDS
benign prostatic hyperplasia (BPH), lower urinary tract symptoms (LUTS), prostate smooth muscle contraction, prostate stromal cell proliferation, thalidomide, α1-adrenoceptors

1 | INTRODUCTION

Lower urinary tract symptoms (LUTS) suggestive of benign prostatic hyperplasia (BPH) are often characterized by voiding symptoms caused by bladder outlet obstruction due to benign prostate enlargement and increased smooth muscle tone. Though it is assumed that prostate smooth muscle contraction is induced by activation of α1-adrenoceptors, hyperplastic prostate stromal cell growth is mediated by dihydrotestosterone. For immediate improvement of symptoms α1-blockers are often applied, whereas 5α-reductase inhibitors have long-term effects through reduction of prostate size. Around 612 million men worldwide were affected by LUTS secondary to BPH in 2018, with annual costs peaking up to five billion USD for medical treatment. The high prevalence combined with high expenses are in strong contrast to the limited efficacy of the available medications. As the gold standard of therapy in LUTS suggestive of BPH, α1-adrenoceptor antagonists improve prostate symptom scores and urinary flow rates (Qmax) by no more than 50%. However, current medications are insufficient, causing high rates of patient noncompliance due to an unfavorable balance between side effects and efficacy, with discontinuation rates peaking up to 65% for mono- and up to 90% for combination therapies. Considering this together with the age-dependency of prevalence and the expected demographic shift in Western countries, improved understanding of these restrictions and novel options with higher efficacy are of high demand.

Even though α1-adrenoceptors have long been regarded as the sole origin of voiding symptoms due to increased prostate smooth muscle tone, cumulative evidence from previous studies suggests, that endothelin-1 and thromboxane A2 (TXA2) may induce maximum levels of prostate contractions in parallel to α1-adrenoceptors. Consequently, these nonadrenergic contractions could maintain urethral obstruction in the presence of α1-blockers and may account for the limited efficacy of α2-blockers. This may explain the high discontinuation rates and high number of nonresponders but could also show the solution to this dilemma. Developing new strategies to address adrenergic and nonadrenergic prostate smooth muscle contraction as well as prostate cell proliferation at once using a single compound seems mandatory.

Thalidomide is a glutamic acid derivative with anti-inflammatory, immunoregulatory, and antiangioproliferative properties. Various effects of thalidomide have been discussed for decades, including its influence on myofibroblast differentiation. Safe administration of thalidomide and its derivative, lenalidomide, for the treatment of erythema nodosum leprosum (ENL) or multiple myeloma is already possible and established. Thalidomide has been used in combination with docetaxel in prostate cancer, making it an already available and safe oral agent and, therefore, its translational value is high. In addition to its antiproliferative role, inhibition of vascular, uterine, and vas deferens smooth muscle by thalidomide has been previously reported. Consequen-tly, similar effects on prostate cell growth and on prostate smooth muscle contraction appear possible. Here, we examined the effects of thalidomide on proliferation and actin organization of cultured prostate stromal cells, and on smooth muscle contractions of human prostate tissues.

2 | METHODS

2.1 | Human prostate tissues

Human prostate tissues were obtained from patients who underwent radical prostatectomy for prostate cancer (n = 62). Patients with previous transurethral resection of the prostate were excluded from the study. 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 the Ludwig-Maximilians University, Munich, Germany. Informed consent was obtained from all patients. All samples and data were collected and analyzed anonymously. Prostates were collected immediately after surgery, followed by macroscopic examination by a pathologist. For macroscopic examination and sampling, the prostate was opened by a single longitudinal cut from the capsule to the urethra. Subsequently, both intersections were checked macroscopically for any obvious tumor infiltration. Tissues were taken from the periurethral zone, considering the fact that most prostate cancers arise in the peripheral zone. In fact, tumor infiltration in the periurethral zone was very rare (found in less than 1% of prostates). Tissue samples showing tumors in the periurethral zone or signs of inflammation in the presence of tumor infiltration upon macroscopic inspection were not subjected to sampling and were not included in this study. In the present study, all specimens were screened and none had to be excluded due to the
above-mentioned criteria. Most importantly, BPH is present in ca.
80% of patients with prostate cancer.27,28 Organ bath studies were
performed immediately after sampling.

2.2 | Tension measurements

Prostate strips (6 × 3 × 3 mm) were mounted in 10-ml aerated (95% O₂ and 5% CO₂) tissue baths (Danish MyoTechnology) 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 thalidomide or dimethyl sulfoxide (DMSO, for controls) were added. Cumulative concentration-response curves for noradrenaline, phenylephrine, methoxamine, endothelin-1, U46619, or frequency response curves induced by electric field stimulation (EFS) were constructed 30 min after addition of thalidomide or DMSO. Effects of thalidomide and corresponding controls were examined in experiments using samples from the same prostate in each experiment. Thus, from each prostate, samples were allocated to the control and thalidomide group within the same experiment. Consequently, both groups in each series had identical group sizes. Moreover, application of DMSO (two chambers) and thalidomide (two chambers) to chambers was changed for each experiment. As two chambers were run for controls and two others for thalidomide 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 % of KCl-induced contractions, as this may correct different stromal/epithelial ratios, different smooth muscle content, varying degree of BPH, and/or any other heterogeneity between prostate samples and patients.29

2.3 | Cell culture

WPMY-1 cells are an immortalized cell line obtained from non-malignant human prostate stroma.30 Cells were obtained from American Type Culture Collection and kept in Roswell Park Memorial Institute 1640 (Gibco) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C with 5% CO₂. Before addition of thalidomide or DMSO (for controls), the medium was changed to an FCS-free medium. Change of medium was performed every day until cells were confluent. After cell counting and determination of the proportionate volume required for further experiments, the cells are transferred to the culture vessels of the respective experiments.

2.4 | Cell proliferation assay

WPMY-1 cells were plated with a density of 50,000/well on a 16-well chambered coverslip (Thermo Fisher Scientific). After 24 h, cells were treated with thalidomide (200 µM, 300 µM) or DMSO and grown for different periods (72 and 168 h). After the aforementioned growth periods, the medium was changed to a 10-mM 5-ethyl-2-deoxyuridine (EdU) solution in an FCS-free medium containing inhibitors or solvent. Twenty hours later, cells were fixed with 3.7% formaldehyde. EdU incorporation was determined using the “EdU Click 555” cell proliferation assay (Baseclick) according to the manufacturer’s instructions. In this assay, incorporation of EdU into DNA is assessed by detection with fluorescing 5-carboxytetramethylrhodamine. Counterstaining of all nuclei was performed with 4,6-diamidino-2-phenylindole. Cells were analyzed by fluorescence microscopy (excitation: 546 nm; emission: 479 nm).

2.5 | Plate colony assay

The plate colony assay is a method to quantify the ability of adherent cells to organize into colonies (>50 cells) after exposure to a specific agent.31,32 After fixing and staining the cells according to the manufacturer’s instruction, the individual cell colonies can be visualized and quantified. The treated cells were exposed to thalidomide for a period of one week and then compared under white light with solvent-exposed controls. Subsequently, the number of cell colonies was calculated, compared to control, and expressed as percentage of the total number of cell colonies.

2.6 | Cell viability assay

The effect of thalidomide on cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich). Cells were grown in 96-well plates (20,000 cells/well) for 24 h before thalidomide or DMSO were added in indicated concentrations (10–300 µM). Subsequently, cells were grown for different periods (72 and 168 h). Separate controls were performed for each period. At the end of this period, 10 µl of [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) from CCK-8 were added, and absorbance in each well was measured at 450 nm after incubation for 2 h at 37°C.

2.7 | 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) was used to detect cells in early apoptosis (annexin V-positive, 7-AAD-negative) and dead cells (annexin V-positive, 7-AAD-positive). Cells were seeded in six-well plates and cultured for 24 h. After the addition of thalidomide and solvent, cells were
incubated for a further 24 h. Subsequently, cells were washed with PBS and resuspended in annexin V binding buffer (BD Biosciences), followed by the addition of 5 µl of APc annexin V and 5 µl 7-AAD reagent to each sample. After incubation in the dark for 15 min at room temperature, 400 µl binding buffer was added to each sample before analysis by flow cytometry.

2.8 Phalloidin staining

For fluorescence staining with phalloidin, cells were grown on Lab-Tek Chamber slides (Thermo Fisher Scientific) with inhibitors or solvent. Staining was performed using 100 µM fluorescein isothiocyanate-labeled phalloidin (Sigma-Aldrich), according to the manufacturer’s instruction. Labeled cells were analyzed using a laser scanning microscope (Leica SP2).

2.9 Data and statistical analysis

Data are presented as means ± standard error of the mean (SEM) with the indicated number (n) of independent experiments. One-way analysis of variance (ANOVA) was used for comparison of whole concentration-response curves and two-way ANOVA was used for comparison of contractions at single concentrations. Friedman test was used for comparison of three or more related groups (e.g., in experiments or included tissues from five or more patients, and the statistical analyses were based on five or more independent experiments or included tissues from five or more patients, and the minimum group size of all groups subjected to statistical tests was n = 5. Moreover, all groups being subjected to statistical tests showed identical group sizes; consequently, any statistical comparisons between groups of different sample sizes, or between groups composed with tissues from different patients were not performed. No data or experiments were excluded from analyses.

3 MATERIALS, DRUGS, AND NOMENCLATURE

Thalidomide (N-(2,6-dioxo-3-piperidinyl)phthalimide) is an inhibitor of tumor necrosis factor α (TNF-α) synthesis and binds cereblon, inhibiting ubiquitin ligase activity. Stock solutions were prepared in DMSO and stored at −20°C until used. Phenylephrine ((R)-3-[1-hydroxy-2-(methylamino)ethyl]phenol), methoxamine (α-(1-Aminooethyl)-2,5-dimethoxybenzyl alcohol), and noradrenaline (4-((1R)-2-Amino-1-hydroxyethyl)-1,2-benzenediol) are selective agonists for α2-adrenoceptors. U46619 ((Z)-7-[[154R,5R,6S]-5-[(E,3S)-3-hydroxyoct-1-enyl]-3-oxacycl[2.2.1]heptan-6-yl]hept-5-enoic acid) is an analog of TXA2 and frequently used as an agonist for TXA2 receptors. Endothelin-1 is a 21-amino acid peptide and potent vasoconstrictor with affinity to the endothelin A (ETα) and B (ETβ) receptors. Aqueous stock solutions of noradrenaline, phenylephrine, and methoxamine were freshly prepared before each experiment. Stock solutions of U46619 were prepared in ethanol and stock solutions of endothelin-1 in water and stored at −80°C until use. Thalidomide was obtained from Tocris, noradrenaline, phenylephrine, and methoxamine were obtained from Sigma-Aldrich, and U46619 and endothelin-1 from Enzo Life Sciences.

3.1 Dosage calculation

Thalidomide is an orally available medication in the treatment of various diseases, including prostate cancer, with dosages of 200–1200 mg per day have been suggested for in vivo clinical trials. For in vitro application dosages of 12.5–387.25 µM have been suggested. To get an approximation, we used an average male body surface area of 2 m² as a reference. Therefore, the clinical dosage of thalidomide is 100–600 mg/m². For viability assay in 96-well plates, the surface area of a single well is 0.32 cm², needing the following equivalent in vitro dose in each well = 100 × 3.2 × 10⁻⁵ = 0.0032 mg. To calculate the concentration of 0.0032 mg of thalidomide for in vitro experiments, we used the molarity formula, where M = mass in grams, MW = molecular weight of the substance, and V = volume of the diluent in liters). 0.0032 mg of thalidomide in 100 µl or 0.0001 L (V) for our in vitro experiments (96-well plate), MW of thalidomide = 258.23 g/mol and m = 0.000032 g. The resultant concentration (molarity) is: 0.0001239 M or 124 µM for 100 mg thalidomide/m² in vivo.

\[ M = \frac{m}{MW} \times \frac{1}{V} = \frac{0.000032 \text{ g}}{258.23 \text{ g/mol}} \times \frac{1}{0.0001 \text{ L}} = 0.0001239 \text{ M} = 124 \text{ µM}. \]
4 | RESULTS

4.1 | Inhibition of WPMY-1 cell proliferation by thalidomide

Thalidomide significantly reduced the relative proliferation rate in WPMY-1 cells (Figure 1). Though 58% ± 1.7% of solvent-treated (72 h) cells showed proliferation, proliferation rate after application of thalidomide (200 µM and 300 µM, 72 h) was reduced to 32% ± 5.1% and 27% ± 4.6%, respectively (p < .0001 for thalidomide vs. control). After incubation for 168 h, proliferation rate for solvent-treated cells was 56% ± 2.3% and for thalidomide-exposed cells 29% ± 5.2% and 24% ± 4.2% for 200 and 300 µM, respectively (p < .01 and p < .0003 for thalidomide vs. control, respectively). Together, this decline was progressive and concentration-dependent (Figure 1A). In a separate series of experiments, lower concentrations of thalidomide were examined. Here, proliferation was observed in 68% ± 2.7% of solvent-treated cells after 72 h, which was significantly reduced after incubation with thalidomide at lower concentrations (10 and 30 µM, 72 h) to 57% ± 2.4% and 51% ± 1.4%, respectively (p < .01 and p < .001 for thalidomide vs. control, respectively). Again, this decline was progressive and concentration-dependent (Figure 1B), but not as pronounced as with higher concentrations.

4.2 | Inhibition of cell colony formation by thalidomide

In a plate colony assay, colony formation was reduced by thalidomide, resulting in relative numbers of colonies amounting to 78% ± 2.6% and 50% ± 3.5% of solvent-exposed controls for 200 and 300 µM thalidomide, respectively (p < .001 and p < .0001 for 200 and 300 µM, respectively, for thalidomide vs. control after 168 h) (Figure 2). The decline in colony formation was concentration-dependent.

4.3 | Regression of actin organization by thalidomide in WPMY-1 cells

We examined the effects of thalidomide on actin organization in cultured WPMY-1 cells. Thalidomide (10–300 µM, 72 h and 200–300 µM, 168 h) caused concentration-dependent degeneration of actin filaments. Actin filaments in solvent-treated control cells were arranged into bundles, forming long and thin protrusions, with elongations from adjacent cells overlapping each other (Figure 3A,B). Thalidomide caused the concentration-dependent loss of actin organization at a concentration of 10–300 µM after 72 and at 200–300 µM after 168 h of incubation, respectively, including regressing degree of actin polymerization and degeneration of protrusions. Thalidomide in
concentrations of 300 μM caused a complete breakdown of actin filament organization after 72 and 168 h, respectively, resulting in a rounded cell shape without protrusions (Figure 3B).

4.4 | Inhibition of prostate smooth muscle contraction by thalidomide

In organ bath experiments, contractions of prostate strips were induced frequency- or concentration-dependently by EFS, noradrenaline, the α1-adrenoceptor-selective agonist phenylephrine and methoxamine, endothelin-1, or U46619. EFS causes neuronal action potentials, leading to contraction by release of endogenous neurotransmitters.39–41

To examine the effects of thalidomide (30–100 μM) on adrenergic contraction, we induced smooth muscle contraction using the adrenergic agonists noradrenaline, methoxamine, and phenylephrine. Noradrenaline-induced adrenergic contractions were decreased up to 60% ± 11.0% with thalidomide for noradrenaline concentrations of 1–100 μM (56% ± 10.5% at 1 μM, p < .02; 60% ± 11.0% at 3 μM, p < .01; 45% ± 7.9% at 10 μM, p < .02; 51% ± 9.3% at 30 μM, p < .01; 44% ± 12.5% at 100 μM, p < .01 for thalidomide vs. control) (Figure 4A). We further examined the effects of thalidomide (100 μM) on methoxamine- and phenylephrine-induced contractions. Thalidomide caused inhibition at 1–100 μM of both methoxamine- and phenylephrine-induced contractions. Methoxamine-induced contractions were decreased up to 64% ± 5.9% (64% ± 5.9% at 3 μM, p < .04; 55% ± 8.5% at 10 μM, p < .02; 47% ± 11.1% at 30 μM, p < .01; 40% ± 16.3% at 100 μM, p < .01 for methoxamine vs. control) (Figure 4B). Thalidomide inhibited phenylephrine-induced contractions by up to 79% ± 6.9% (69% ± 11.7% at 3 μM, p < .03; 66% ± 7.4% at 10 μM, p < .01; 79% ± 6.8% at 30 μM, p < .001; 79% ± 6.9% at 100 μM, p < .001 for thalidomide vs. control) (Figure 4C). In a separate series of experiments including phenylephrine-induced contractions, we applied a lower thalidomide concentration (30 μM), and still observed inhibition of phenylephrine-induced adrenergic contractions of up to 37% ± 17.0% with overall p < .03 for thalidomide versus control (Figure 4D).

The effect of thalidomide (30–100 μM) on nonadrenergic contraction was demonstrated by using the nonadrenergic

![FIGURE 2](image-url)  
**Figure 2**  Inhibition of prostate stromal cell colony formation by thalidomide. Shown is the relative number of colonies after 168 h (means ± SEM) from a series using cell cultures from n = 5 independent experiments. The cells were either allocated to a control or a thalidomide group (#p < .05) and incubated 168 h. Shown are exemplary images of colony formation after 168 h (left), and quantification of all experiments (right) [Color figure can be viewed at wileyonlinelibrary.com]

![FIGURE 3](image-url)  
**Figure 3**  Inhibition of prostate stromal cell actin organization by thalidomide (10–300 μM). Shown are actin filaments after 72 and 168 h from a series using cell cultures from n = 5 independent experiments for each concentration, respectively. The cells were either allocated to a control or a thalidomide group and incubated 72 or 168 h. Actin filaments were visualized by phalloidin staining and fluorescence microscopy, whereas the nuclei were visualized using 4′,6-diamidino-2-phenylindole staining. Shown are exemplary images after 72 and 168 h [Color figure can be viewed at wileyonlinelibrary.com]
agonists endothelin-1 and the thromboxane A2 analog U46619. At a concentration of 30 and 100 µM, thalidomide significantly inhibited endothelin-1-induced contractions up to 27% ± 2.5% with overall p < .05 and 74% ± 19.2%, respectively (50% ± 12.9% at 0.3 µM, p < .04; 7% ± 19.2% at 1 µM, p < .01; 56% ± 20.5% at 3 µM, p < .01 for thalidomide vs. control) (Figures 5A and 5C).

U46619–induced contraction was inhibited up to 36% ± 8.8% by 30 µM thalidomide with overall p < .05 and 106% ± 43.8% by 100 µM thalidomide (106% ± 43.8% at 1 µM, p < .05; 78% ± 22.5% at 3 µM, p < .01; 89% ± 9.0% at 10 µM, p < .001; 81% ± 9.9% at 30 µM, p < .001 for thalidomide vs. control) (Figures 5B and 5D).

Finally, we investigated the effect of thalidomide on neurogenic contraction induced by EFS. Significant inhibition up to 72% ± 13.5% using 100 µM thalidomide in EFS-induced contractions induced by 2–32 Hz (100 µM thalidomide: 70% ± 15.2% at 16 Hz, p < .01; 72% ± 13.5% at 32 Hz, p < .001 for thalidomide vs. control) (Figure 6A) and up to 37% ± 10.5% with overall p < .02 was observed using 30 µM thalidomide (Figure 6B).

### 4.5 Viability of thalidomide in WPMY-1 cells

Effects of thalidomide on viability of WPMY-1 cells were assessed by CCK-8 assay. For each concentration and time, independent experiments with n = 5 for each set of experiments were performed. Low concentrations of thalidomide (10 and 30 µM) were applied for 24–72 h, and did not reduce survival. After application of thalidomide for 24 h survival was 95% ± 0.5% (p = .68) and 92% ± 0.5% (p = .27) of solvent-treated controls for 10 and 30 µM thalidomide, respectively. After 48 h survival was 92% ± 1.5% (p = .61) and 89% ± 1.7% (p = .23), and after 72 h survival was 94% ± 1.3% (p = .60) and 89% ± 2.2% (p = .18) of solvent-treated controls for 10 and 30 µM thalidomide, respectively (Figure 7A). Similarly, we could not detect any significant decrease in survival after incubation for 72 and 168 h, respectively, even for higher concentrations (100–300 µM thalidomide). After application of thalidomide for 72 h, survival was 94% ± 3.6%, 91 ± 4.0% and 91% ± 4.2% of solvent-treated controls for 100, 200, and 300 µM, respectively (p = 1.0 for 100 µM, p = 1.0 for 200 µM, and p = .72 for 300 µM thalidomide vs. control) (Figure 7). Even longer application...
FIGURE 5  Effects of thalidomide on nonadrenergic contraction of human prostate tissues. Contractions were induced by endothelin-1 and the thromboxane A2 analog U46619 after addition of thalidomide (30–100 µM) or dimethyl sulfoxide (DMSO) for controls. To eliminate heterogeneities due to individual variations, different degrees of BPH, or other varying smooth muscle content, tensions have been expressed as percentages (%) of contraction by high molar KCl, being assessed before application of inhibitors or solvent. Data are means ± SEM from series with tissues from $n=6$ patients for endothelin-1 and $n=7$ patients for U46619 after incubation with 30 µM thalidomide (A and B, respectively) and $n=5$ patients for endothelin-1 and $n=7$ patients for U46619 after incubation with 100 µM thalidomide (C and D, respectively). Samples from each patient were allocated to both groups within one diagram so that both groups in each diagram had identical group sizes. $p$ Values include values for each single concentration between both corresponding groups, being indicated by symbols ($#p < .05$ for DMSO vs. thalidomide).

FIGURE 6  Effects of thalidomide on electric field stimulation (EFS)-induced neurogenic, contraction of human prostate tissues. Contractions were induced by EFS, after addition of thalidomide (30–100 µM) or dimethyl sulfoxide (DMSO) for controls. To eliminate heterogeneities due to individual variations, different degrees of BPH, or other varying smooth muscle content, tensions have been expressed as percentages (%) of contraction by high molar KCl, being assessed before application of inhibitors or solvent. Data are means ± SEM from series with tissues from $n=8$ and $n=5$ patients for EFS (frequency 2–32 Hz) after incubation with 30 µM and 100 µM thalidomide, respectively (A and B). Samples from each patient were allocated to both groups within one diagram so that both groups in each diagram had identical group sizes. $p$ Values include values for each single concentration between both corresponding groups, being indicated by symbols ($#p < .05$ for DMSO vs. thalidomide).
for 168 h did not reduce survival, which was 98% ± 3.9%, 94% ± 2.9%, and 87% ± 7.8% of controls for 100, 200, and 300 µM, respectively (p = 1.0 for 100 µM, p = 1.0 for 200 µM, and p = 1.0 for 300 µM thalidomide vs. control) (Figure 7B).

4.6 | Effects of thalidomide on apoptosis

Effects of thalidomide on early apoptosis and on cell death was assessed by flow cytometry analysis for annexin V and 7-AAD, where annexin V-positive, 7-AAD-negative cells represent cells in early stages of apoptosis, and annexin V-positive, 7-AAD-negative cells represent dead cells (which may result either from apoptosis or necrosis). Thalidomide (300 µM, 72 h) did not increase the number of cells in apoptosis or of dead cells (Figure 8).

5 | DISCUSSION

Using thalidomide for the first time in the context of LUTS, our findings suggest, that it may be possible to target both smooth muscle contraction and prostate growth at once by using a single compound. Both processes are critical for etiology and therapy of voiding symptoms in patients with LUTS suggestive of BPH, but monotherapies still fail to address these mechanisms simultaneously.1,2,42 Available medical therapy options for LUTS suggestive of BPH certainly improve the clinical situation for many patients. However, insufficient efficacy, disappointing responder rates, and high discontinuation rates provide clear limitations of current pharmacotherapy.1,2,8,42,43 Although drugs addressing smooth muscle contraction and hyperplastic growth in the prostate at once may be attractive candidates for the treatment of male LUTS, this possibility has rarely been considered. Here, we examined the effects of thalidomide on prostate smooth muscle contraction and growth of prostate stromal cells. On the basis of these observations in vitro, it may be expected that thalidomide induces urodynamic effects in vivo, which could even outrank those of α₁-blockers and 5α-reductase inhibitors. Though available compounds for medical treatment of male LUTS either address α₁-adrenergic contraction or prostate size, thalidomide was capable of decreasing adrenergic and nonadrenergic contraction, as well as prostate stromal cell growth at once. We are aware that any efficacy of thalidomide for improvement of LUTS needs to be confirmed in vivo, and that application may be limited by side-effects. Thalidomide, although withdrawn from the market in the 1960s for causing notorious birth defects, has been Food and Drugs Administration-approved for treating ENL in 1998 and further continues to expand its therapeutic value.12,14,18,19,44

Apart from anti-inflammatory and immunoregulatory effects, thalidomide also shows antiangioproliferative properties.12–14 Thalidomide seems to exert and regulate the expression and synthesis of vascular endothelial growth factor, transforming growth factor β (TGF-β) and α-smooth muscle actin (α-SMA) in other organ systems, such as liver and lung.15–17 Such growth factors, along with androgens and inflammatory mediators, appear to be crucial regulators of prostate stromal cell growth.45,46 In particular, TGF-β seems to play an important role. As any effect on proliferation of prostate stromal cells may be interesting for application in LUTS secondary to BPH, where hyperplastic growth often results in symptoms, we examined effects of thalidomide on proliferation of WPMY-1 cells. Thalidomide significantly reduced the proliferation rate in WPMY-1 cells at different concentrations (10–300 µM), which was assessed by an EdU assay. In parallel to the diminished proliferation rate, we observed, that thalidomide decreased the formation of cell colonies. With plate colony assay, individual cell colonies can be visualized and quantified,31,32 representing an alternative indicator not only of individual single-cell proliferation but also of collective growth.47 However, our findings may be limited due to the fact that we used an immortalized and karyotypically abnormal cell line (WMPY-1) and, therefore, should be interpreted with care.30
In addition to the effect on cell proliferation and colony formation, we observed an effect of thalidomide on the actin cytoskeleton. Though the precise mechanisms underlying effects of thalidomide on proliferation remain to be identified in future studies, the breakdown of actin organization may well account for decreased contractility in organ bath experiments. As the correct organization of actin filaments is a prerequisite for smooth muscle contraction, any modulation of the actin cytoskeleton could be assumed to translate to decreased smooth muscle contractility in the prostate, which, in contrast to α₂-blockers, could affect not only α₁-adrenergic but also adrenergic and nonadrenergic contraction. Actin polymerization is indispensable for smooth muscle contraction and turned out to be susceptible to thalidomide at a wide range of concentrations (10–300 µM) in WPMY-1 cells. Consequently, thalidomide could be an important regulator or mediator of smooth muscle contraction. Recently, effects of thalidomide on murine uterine, vas deferens, and vascular smooth muscle contraction have been reported.\(^{21,23}\) Considering this combined with our results obtained from cell culture, we speculated that thalidomide may inhibit prostate smooth muscle contraction in human prostate tissue using an organ bath.

To the best of our knowledge, this study is the first demonstrating inhibition of prostate smooth muscle contraction by thalidomide using intact tissues. Thalidomide was applied in concentrations of 30–100 µM in our organ bath experiments, corresponding to the concentrations used by the above-mentioned studies. We demonstrated significant and extensive inhibition of α₁-adrenergic as well as nonadrenergic and neurogenic prostate smooth muscle contraction. The actions of thalidomide, especially on smooth muscle contraction, may be novel. However, thalidomide has the potential to work through many different pathways and its inhibition of cell growth may not be specific to the human prostate.\(^{15,16,48}\)

The role of prostate growth and smooth muscle contraction for development and medical therapy of male LUTS may impart a translational value to our current findings. Certainly, and according to the history of thalidomide, effects on cellular survival in other cell types need to be considered in preclinical studies like the current

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**FIGURE 8** Apoptosis and cell death in prostate stromal cells after stimulation with thalidomide. Flow cytometry was performed after cells were treated for 72 h with dimethyl sulfoxide or thalidomide (300 µM). Subsequently, the numbers of cells being in apoptosis ('early apoptosis': annexin V-positive, 7-AAD-negative), and of dead cells (reflecting from apoptosis and/or necrosis; annexin V-positive, 7-AAD-positive) were assessed by flow cytometry. Shown are means ± SEM from a series of \(n = 5\) independent experiments, and representative single experiments [Color figure can be viewed at wileyonlinelibrary.com]
one, and are crucial in in vivo settings. However, thalidomide does not act generally cytotoxic in any cell type. Using a CCK-8 kit, we assessed cell viability after incubation of WPMY-1 cells with thalidomide. Significantly reduced viability could not be detected at any incubation time or concentration. Accordingly, we did not observe effects of thalidomide on apoptosis or induction of cell death by thalidomide even at higher concentrations. Since being withdrawn from the market in the 1960s thalidomide has been reintroduced in 1998 and is expanding its therapeutic value in treating various diseases.

It is justified to assume urodynamic efficacy in vivo resulting from simultaneous inhibition of adrenergic and nonadrenergic prostate smooth muscle contractions as well as inhibition of prostate stromal cell growth: First, there is increasing evidence that benefits from α1-blockers are limited, second, the maximum level of prostate smooth muscle tone may be completely induced independently from α1-adrenoceptors, and third, inhibition of prostate smooth muscle tone in the presence of untreated benign prostatic enlargement may be insufficient. Even new and highly selective α1A-blockers may not be superior to previous medications, which was recently exemplified with the introduction of silodosin. Even though silodosin has a higher subtype-selectivity for α1A-adrenoceptors than previous α1-blockers, and induces prostatic smooth muscle relaxation in the absence of cardiovascular side effects (which are mainly mediated via α1B-adrenoceptors), its efficacy in LUTS suggestive of BPH turned out to be just similar to other α1-blockers. However, due to the rather prominent discrepancy between oral dosages and in vitro concentrations, our results have to be interpreted with care and warrant further clinical evaluation.

Nevertheless, the demand for alternative treatment options is high and may be found by introducing new compounds derived from drug classes other than α1-blockers. A successful example may be the introduction of phosphodiesterase-type 5 inhibitors, such as tadalafil, which were recently introduced for the treatment of LUTS suggestive of BPH. In combination with other drugs, they may show efficacies outranking previous monotherapies. As thalidomide is often attributed a similarity to phosphodiesterase inhibitors, it may be a very promising new compound in the treatment of LUTS.

6 | CONCLUSIONS

From a translational point of view, thalidomide is a promising candidate with regard to future evaluation of urodynamic effects in vivo. The highest efficacy regarding the improvement of LUTS suggestive of BPH may be expected from inhibitors targeting α1-adrenergic and neurogenic, as well as endothelin- and TXA2-induced, that is, non-adrenergic prostate smooth muscle contractions, and prostate stromal cell growth at once. This evidence is corroborated by our observations that thalidomide significantly inhibits SMA polymerization. As far as we know the application of thalidomide in the context of multiple myeloma and ENL is safe and effective, which also stands in unison with our findings, that thalidomide does not decrease cell viability or promote apoptosis. There are few side effects from thalidomide and actions in various cell types appear possible so that it should not be administered to actively reproducing men. Moreover, the inhibition of neurogenic contractions by thalidomide observed in our study appeared to be robust and strong, so that the translational relevance is obvious and appears promising. Considering these aspects together, thalidomide may be an attractive compound to be transferred to in vivo studies addressing urodynamic effects.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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