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Honokiol, a constituent of *Magnolia* species, inhibits adrenergic contraction of human prostate strips and induces stromal cell death

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Purpose: Smooth muscle contraction and prostate growth are important targets for medical therapy of lower urinary tract symptoms (LUTS) in patients with benign prostatic hyperplasia. Honokiol and Magnolol are lignan constituents of *Magnolia* species, which are used in traditional Asian medicine. Here, we examined effects of honokiol and magnolol on contraction of human prostate tissue and on growth of stromal cells.

Methods: Prostate tissues were obtained from radical prostatectomy. Contraction of prostate strips was examined in organ bath studies. Effects in stromal cells were assessed in cultured immortalized human prostate stromal cells (WPMY-1). Ki-67 mRNA was assessed by reverse transcription-polymerase chain reaction, and proliferation by a fluorescence 5-ethynyl-2′-deoxyuridine assay.

Results: Honokiol (100 μM) reduced noradrenaline-induced contractions, which was significant at 10- to 100-μM noradrenaline. Honokiol reduced phenylephrine-induced contractions, which was significant at 3- to 100-μM phenylephrine. Honokiol reduced electric field stimulation-induced contractions very slightly. In WPMY-1 cells, honokiol (24 hours) induced cell death. Magnolol (100 μM) was without effects on contraction, and cellular viability.

Conclusions: Honokiol inhibits smooth muscle contraction in the human prostate, and induces cell death in cultured stromal cells. Because prostate smooth muscle tone and prostate growth may cause LUTS, it appears possible that honokiol improves voiding symptoms.

Keywords: Magnolia, Honokiol, Prostatic hyperplasia, Lower urinary tract symptoms, Adrenergic alpha-1 receptors

INTRODUCTION

Medical therapies of lower urinary tract symptoms (LUTS) target either smooth muscle contraction in the lower urinary tract, or prostate growth [1]. In fact, exaggerated α_1-adrenoceptor-mediated contraction, and enlargement of the prostate may both contribute to bladder outlet obstruction and LUTS in patients with benign prostatic hyperplasia (BPH) [2]. Consequently, options for medical treatment comprise application of α_1-blockers to induce prostate smooth muscle relaxation, and 5α-reductase inhibitors to reduce prostate growth and volume [1].

In addition to α_1-blockers and 5α-reductase inhibitors, the use of phytotherapeutics is extremely widespread at least in Western Europe and the United States [3,4]. Several preparations containing combined plant extracts are available without prescription, and still represent an important mainstay of LUTS therapy [5]. Their use is widespread; it has been estimated that phytotherapeutics may still reach up to 30 % of total expenses for medical LUTS therapy, which amounted to 4.8 billion USD worldwide in 2009 [3,6,7]. Although their effects are controver-

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sially discussed, several clinical studies meeting World Health Organization (WHO) criteria demonstrated improvements of LUTS, urinary flow rate, and postvoid residual volume [5].

Honokiol and magnolol are neolignan compounds of different *Magnolia* species [8]. Bark and flowers from *Magnolia obovata* and *Magnolia officinalis* have been used to date in traditional Chinese and Japanese herbal medicine for treatment of gastrointestinal disorders, anxiety, and allergic disease [8]. Following isolation and identification of honokiol and magnolol from magnolia extracts, their antitumor activity has been recognized and examined in experimental models [8]. Recent studies suggested that they may induce smooth muscle relaxation in the cardiovascular system, airways, and gastrointestinal tract [9-16]. However, their actions in the lower urinary tract are unknown to date. Here, we investigated the effects of honokiol and magnolol on contraction of human prostate tissue.

MATERIALS AND METHODS

1. Human prostate tissue

Human prostate tissues were obtained from April 2013 to September 2013 from patients undergoing radical prostatectomy (n=38) for prostate cancer, but without previous transurethral resection of the prostate (TURP). The 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. Approval did not allow acquisition or storage of any patients' data, so that all samples were treated anonymously. Consequently, data analysis with relation to patients' characteristics (e.g., age) was not possible, what may be viewed as a limitation of the study. Tissues in our study were exclusively taken from the periurethral zone. Most prostate tumors are located to the peripheral zone. Consequently, the extent of malignant areas (if any) in our samples may be neglected. Tissue samples did not exhibit macroscopical signs of neoplasia, cancer, or inflammation. Samples were immediately taken after prostatectomy and subsequent macroscopical pathological examination. Organ bath studies were performed immediately after sampling.

2. Tension measurements

Prostate strips (6 mm×3 mm×3 mm) were mounted in 10 mL aerated (95% O_2 and 5% CO_2) tissue baths (Föhr Medical Instruments, Seeheim, Germany), containing Krebs-Henseleit solution (37°C, pH 7.4). Preparations were stretched to 0.5 g and left to equilibrate for 45 minutes. 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 (0.5 g) was attained. After the equilibration period, maximum contraction induced by 80mM KCl (Krebs-Henseleit solution where NaCl was exchanged by KCl) was assessed. Subsequently, chambers were washed three times with Krebs-Henseleit solution for a total of 30 minutes. Cumulative concentraction response curves for noradrenaline or phenylephrine (both from Sigma-Aldrich, Munich, Germany) were created after addition of 100-μM honokiol, 100-μM magnolol (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or an equivalent volume of solvent (dimethylsulfoxide, DMSO). Fequency response curves induced by electric field stimulation (EFS) were created before and after addition of inhibitors or solvent (DMSO for honokiol, water for tamsulosin, and DMSO for the combination of both). Inibitors or DMSO were applied 45 minutes before concentration or frequency response curves.

3. Cell culture

WPMY-1 cells are an immortalized cell line obtained from nonmalignant human prostate stroma. Cells were obtained from American Type Culture Collection (Manassas, VA, USA), and kept in Rosewell Park Memorial Institute 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C with 5% CO_2. Before stimulation with honokiol (100 μM, 24 hours) or magnolol (100 μM, 24 hours), the medium was changed to a FCS-free medium. At the end of the experiment, cells were microscoped and pictures were taken using the AxioCam (Zeiss, Oberkochen, Germany).

4. Determination of mRNA expression by real-time polymerase chain reaction

Total RNA was isolated from WPMY-1 cells using the RNeasy mini Kit (Qiagen, Hilden, Germany). After reverse transcription with AMV reverse transcriptase (Promega, Fitchburg, WI, USA), real-time reverse transcription-polymerase chain reaction was performed to assess Ki-67 and ß-actin expression with QuantiTect Primer Assays (Qiagen) using a LightCycler Instrument (Roche Diagnostics, Rotkreuz, Switzerland).

5. Cell proliferation assay

WPMY-1 cells were plated with a density of 50,000/well on a 16-well chambered coverslip (Thermo Scientific, Waltham, MA, USA). After 24 hours, cells were treated with honokiol (100μM) or magnolol (100μM) in FCS-free medium. After further 24 hours, the medium was changed to a 10mM 5-ethy-

nyl-2'-deoxyuridine (EdU) solution in FCS-free medium containing honokiol or magnolol. After 20 hours, cells were fixed with 3.7% formaldehyde. EdU incorporation was determined using the "EdU-Click 555" cell proliferation assay (Baseclick, Tutzing, Germany) 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 flourescence microscopy (excitation, 546 nm; emission, 479 nm).

6. Drugs and solutions

Honokiol (2-(4-hydroxy-3-prop-2-enyl-phenyl)-4-prop-2-enyl-phenol) and magnolol (4-Allyl-2-(5-allyl-2-hydroxyphenyl)phenol) are biphenyl lignans, isolated from *Magnolia* species extracts. Stock solutions (10mM) were prepared with DMSO, and kept at −20°C until use. Phenylephrine ((R)-3-[-1-hydroxy-2-(methylamino)ethyl]phenol) is an agonist of the α_1-adrenoceptor. Aqueous stock solutions of phenylephrine and noradrenaline (10mM) were freshly prepared before each experiment.

7. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) with the indicated number of experiments. Two-tailed Student *t*-test was used for paired or unpaired observations. Values of $P<0.05$ were considered statistically significant. Concentrations producing the half-maximal responses (EC_{50} values) for contractile agonists were calculated using GraphPad Prism ver. 6 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

1. Adrenergic contraction

Noradrenaline and the α_1-adrenergic agonist phenylephrine induced concentration-dependent contractions of human

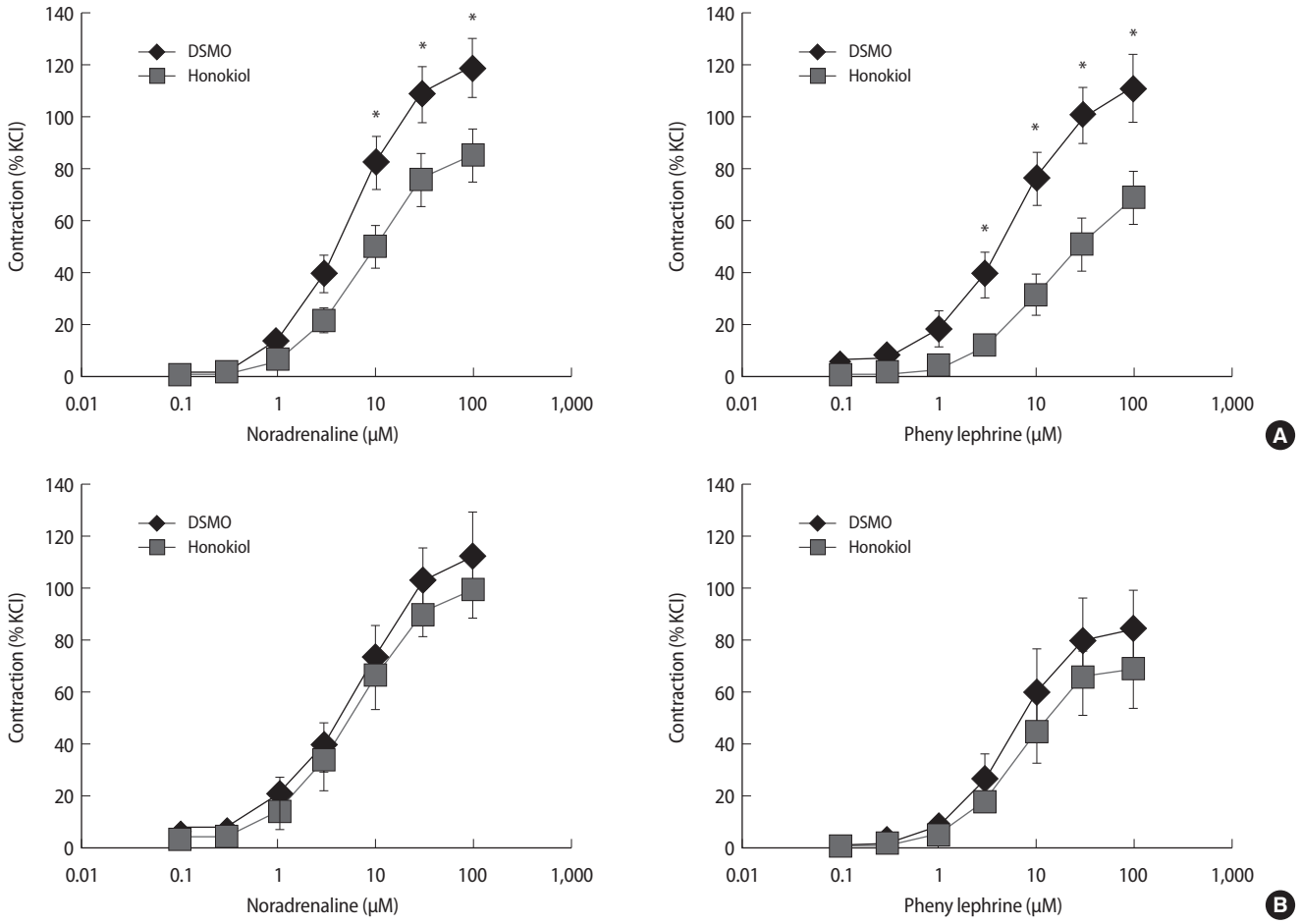

Fig. 1. Effects of honokiol (A) and magnolol (B) on noradrenaline- and phenylephrine-induced contractions of human prostate strips. Contractions were assessed after application of solvent (dimethylsulfoxide, DMSO), and honokiol (100μM) or magnolol (100μM). Tensions are expressed as % of contraction induced by 80mM KCl. Data are mean ± standard error of the mean from experiments with prostates from n=10 patients for noradrenaline/honokiol, n=8 patients for phenylephrine/honokiol, n=6 patients for noradrenaline/magnolol, or n=5 patients for phenylephrine/magnolol. *$P<0.05$ for DMSO vs. honokiol.

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prostate strips (Fig. 1). These were reduced by honokiol (100 μM). Significant inhibition of noradrenaline-induced contraction by honokiol was observed at 10μM (P=0.016 for honokiol vs. DMSO), 30μM (P=0.034), and 100μM (P=0.035) noradrenaline (Fig. 1A). Significant inhibition of phenylephrine-induced contraction by honokiol was observed at 3μM (P=0.013), 10μM (P=0.0049), 30μM (P=0.0028), and 100μM (P=0.015) phenylephrine (Fig. 1A). In addition to inhibition of force generation, honokiol significantly increased the EC_{50} values for noradrenaline (P=0.022 for honokiol vs. DMSO) and phenylephrine (P=0.025) (Table 1).

In contrast, application of magnolol (100μM) caused only slight inhibition of noradrenaline- and phenylephrine-induced contractions, without being significant (Fig. 1B). EC_{50} values for noradrenaline or phenylephrine was not changed by magnolol (Table 1).

2. EFS-induced contraction

EFS induced frequency-dependent contractions of human prostate strips (Fig. 2). Contractions were virtually identical before and after application of DMSO (Fig. 2). In contrast, contractions induced by 8, 16, and 32 were slightly reduced after application of honokiol, without being significant (Fig. 2).

Table 1. EC_{50} values for noradrenaline- and phenylephrine-induced contractions of human prostate strips, after application of honokiol, magnolol, or solvent (DMSO)

	$LogEC_{50}$ noradrenaline (M)	$LogEC_{50}$ phenylephrine (M)
Honokiol	n=10	n=8
DMSO	−5.205±0.049	−5.159±0.099
Honokiol	−4.926±0.097[a)]	−4.622±0.19[a)]
Magnolol	n=6	n=5
DMSO	−5.217±0.186	−5.2±0.139
Magnolol	−5.18±0.155	−5.13±0.067

Values are presented as mean±standard error of the mean.
DMSO, dimethylsulfoxide.
[a)] P<0.03 vs. corresponding DMSO control.

3. Effects of honokiol on WPMY-1 cells

Application of honokiol (100μM) for 24 hours induced death of cultured WPMY-1 cells (Fig. 3). Microscopic examination revealed that cells were completely destroyed by treatment with honokiol.

4. Effects of magnolol on WPMY-1 cells

Application of magnolol (100μM) for 24 hours was without effect on cell cycle of WPMY-1 cells. In a fluorescent EdU assay, 53% of cells showed proliferation after application of magnolol, while 57% showed proliferation in control samples (Fig. 4). Magnolol did not change the mRNA expression of the proliferation marker, Ki-67 (Fig. 4).

DISCUSSION

Our findings demonstrate that honokiol may interfer with contraction and cell cycle in the human prostate. Induction of smooth muscle relaxation by α_1-adrenoceptor blockers, and inhibition of prostate growth by 5α-reductase inhibitors are important strategies for medical LUTS therapy [17]. However, a single substance targeting prostate contraction and growth at once has not been approved to date. Currently, combination therapies including α_1-blockers and 5α-reductase inhibi-

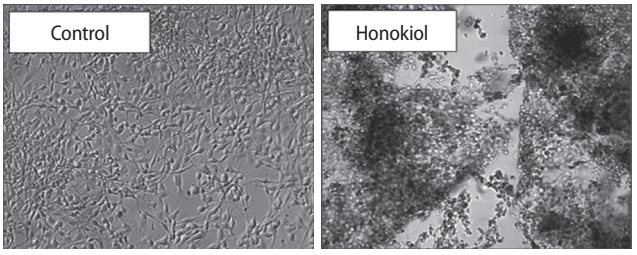

Fig. 3. Effect of honokiol on WPMY-1 cells. Honokiol (100μM) was applied to cultured WPMY-1 cells for 24 hours. Shown are representative pictures after application of honokiol or from control samples without honokiol, from a series of 3 independent experiments with identical results.

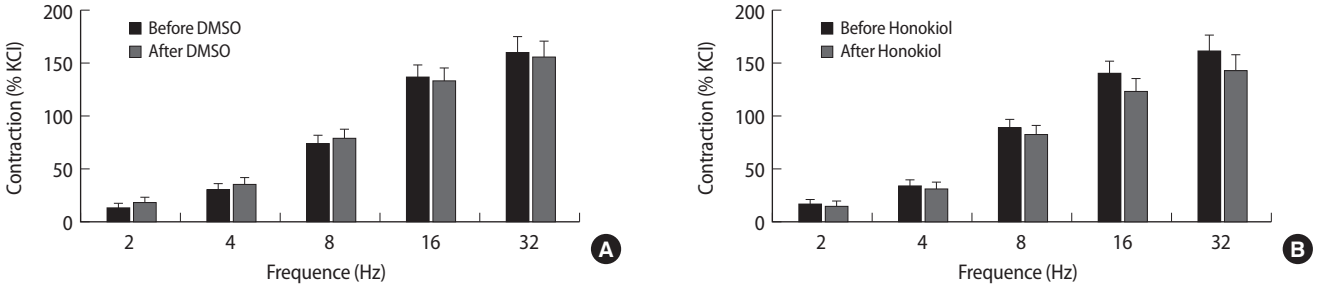

Fig. 2. Effects of honokiol (100μM) on electric field stimulation-induced contractions of human prostate strips. Contractions were compared before and after application of solvent (dimethylsulfoxide, DMSO) (A) or honokiol (B) to prostate strips. Tensions are expressed as % of contraction induced by 80mM KCl. Data are mean±standard error of the mean from experiments with prostates from n=9 patients.

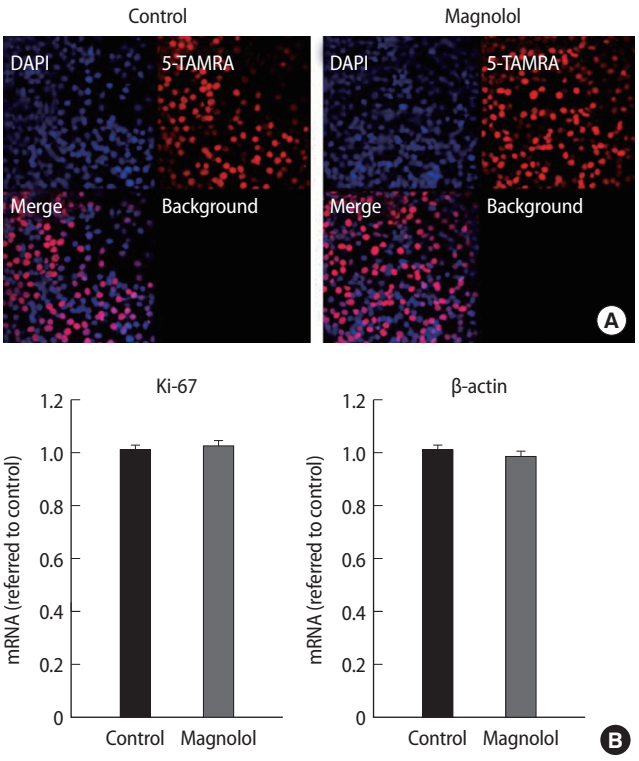

Fig. 4. Effects of magnolol on cell cycle of WPMY-1 cells. Magnolol (100μM) was applied to cultured WPMY-1 cells for 24 hours. (A) Proliferation was assessed by a 5-ethynyl-2′-deoxyuridine assay, where red-orange fluorescence of 5-carboxytetramethylrhodamine (5-TAMRA) in nuclei indicates proliferation. Counterstaining of all nuclei was performed with 4′,6-diamidino-2-phenylindole (DAPI). (B) mRNA expression of the proliferation marker, Ki-67, and of β-actin was assessed by quantitative reverse transcription-polymerase chain reaction. Content in magnolol samples was referred to control samples, which were set to 1. Shown are representative pictures (A) or mean±standard error of the mean (B) from series with 3 independent experiments.

tors are applied to patients, where monotherapy with α_1-blockers is insufficient [17]. However, these combinations do not only provide increased benefits, but also additive side effects [17]. Therefore, a single substance addressing both components at once together with low side effects may be appreciated. Numerous studies described inhibition of different smooth muscle preparations and inhibition of cellular growth and proliferation by honokiol and magnolol. This prompted us to investigate effects of honokiol and magonolol on prostate smooth muscle contraction, and growth of stromal cells.

In our tension measurements, honokiol caused significant inhibition of phenylephrine- and noradrenaline-induced contractions, and increased EC_{50} values for both agonists. In the human prostate, α_1-adrenoceptor-mediated contraction critically determines smooth muscle tone [2]. A minor effect of honokiol was observed at EFS-induced contractions with high frequencies. Inhibition of smooth muscle contraction by honokiol has been previously reported from the guinea pig ileum, porcine trachea, rat uterus, and from rat aortic rings [9-12]. To the best of our knowledge, our study using human prostate tissue is the first showing inhibition of smooth muscle contraction by honokiol in a smooth muscle preparation of human origin.

In WPMY-1 cells, a line of stromal cells obtained from a nonmalignant human prostate, application of honokiol induced cell death. This is in line with previous findings, where honokiol was studied in prostate cancer or smooth muscle cells [18-22]. Thus, honokiol induced apoptosis or cell cycle arrest in different lines of prostate cancer cells [19,20,22]. Similarly, it induced apoptosis and cell cycle arrest in cultured vascular smooth muscle cells [18,21]. Proliferation of stromal cells is critical for prostate growth and enlargement in BPH [23]. Together, this suggests that *in vivo* application of honokiol may reduce growth and volume of the hyperplastic prostate.

Contrary to previous findings from other organs and species, magnolol caused no significant inhibition of smooth muscle contractions in human prostate strips. Inhibition of smooth muscle contraction by magnolol was reported from preparations of rat and guinea pig colon, guinea pig ileum, rat aortic rings, rat uterus, and porcine trachea [9-11,13-16]. Similarly, magnolol was without effect on cell cycle of WPMY-1 cells, although it induces apoptosis or cell cycle arrest in cultured prostate cancer and smooth muscle cells [24-28]. Based on studies in the cardiovascular system, it has been previously assumed that effects of magnolol may be cell type- and dosage-specific [29]. Obviously, limitations of magnolol actions are not confined to the cardiovascular system, but also occur in the lower urinary tract, where magnolol acts on smooth muscle contraction in the rat uterus but not in the human prostate [11].

The dual actions of honokiol on contraction and cell cycle in the prostate appears attractive for therapy of LUTS suggestive of benign prostatic obstruction (BPO). Whether honokiol induces improvement of voiding symptoms can only be assessed *in vivo*. Because prostate-dependent mechanisms can not be studied in wide-spread rodent models, urodynamic effects of honokiol should preferentially examined in clinical proof-of-concept studies. To the best of knowledge, tests in preclinical models did not provide any clues, which may exclude studies with oral uptake of honokiol. In previous studies, which adressed antitumor activity and pharmacokinetics in different animal models, honokiol-containing preparations were mostly applied intravenously, but also orally or even rectally in rats [30]. After rectal application, plasma concen-

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trations of honokiol may be up to six times higher compared to oral application, what may be interesting for applications in LUTS therapy [30]. Meanwhile, liposomal formulations of honokiol including polyethylene glycol coated (PEGylated) liposomal honokiol have been and are still developed and tested for *in vivo* applicatons for anticancer therapies [30-32]. Toxicologic and mutagenic studies in preclinical models suggested that honokiol may be safe and is not genotoxic [30].

For treatment of LUTS, different preparations containing single or mixed plant extracts are available [1,5]. Their high popularity may raise from their easy availability, but also from disappointment of established clinical options. It has been estimated that 36%-45% of patients are not satisfied from treatment with α_1-blockers or 5α-reductase inhibitors, raising the need for ablative treatments [17]. Symptoms, assessed as "International Prostate Symptom Score" (IPSS) are improved to 30%–50% by α_1-blockers and to 15%–30% by 5α-reductase inhibitors, but to 10%–34% by placebos [1,2,6]. Similarly, maximal flow rate (Qmax) is increased to 15%–40% by α_1-blockers, while increases up to 27% were observed in response to placebo [1,2,6]. Finally, side effects may account for discontinuation of medical therapy, with varying rates for α_1-blockers or 5α-reductase inhibitors [6]. Together, this raises a high interest for alternative options.

LUTS treatment with plant extracts is still controversially discussed. Several clinical studies meeting international WHO-BPH standards addressed effects of phytotherapy in patients with LUTS suggestive of BPO, with adverse results. Preparations containing plant extracts were often indistinguishable from placebos, with few exceptions [33-37]. Positive findings mostly concerned IPSS, while improvements of Qmax were found rarely [35,36]. Based on these findings, neither a recommendation, nor a clear rejection/dismissial of phytotherapies was given [1,5].

In conclusion, Honokiol inhibits smooth muscle contraction in the human prostate, and induces cell death in cultured stromal cells. Because prostate smooth muscle tone and prostate growth may cause LUTS, it appears possible that honokiol may improve voiding symptoms in patients with BPH.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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