이학박사학위논문

식물소재로부터 전립선 비대를 조절하는 생물학적 활성 HX109 의 분리와 이에 대한 분자생물학적 분석

Isolation of Biological Activities Regulating Prostatic Hyperplasia, HX109, from Plant Sources and Its Molecular Biological Characterization

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ABSTRACT

Isolation of Biological Activities Regulating Prostatic Hyperplasia, HX109, from Plant Sources and Its Molecular Biological Characterization

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Benign prostatic hyperplasia (BPH) is a common disease in the elderly male population throughout the world. The etiology of BPH has yet to be fully elucidated, but progression of the disease involves various factors including aging, hormonal changes, metabolic syndrome, oxidative stress, and inflammation. Although drugs such as 5α -reductase inhibitors (5ARI) and a-blocker are currently in use, they have several side effects and there is a need for the development of safe, effective, and long-lasting therapeutic agents.

Since BPH is caused by multiple factors, plants may provide an interesting source for developing therapeutic agents. Plants containing multiple component have an advantage because they interact with multiple cellular targets. Therefore, HX109, a botanical formulation prepared from three plants (*Taraxacum officinale*, *Cuscuta australis*, and *Nelumbo nucifera*) was developed to target BPH. An ethanol extract was prepared from a mixture of these three plants, and its quality

was controlled through cell-based bioassays and by quantification of several marker compounds by high-performance liquid chromatography (HPLC). In this study, it was hypothesized that HX109 might exhibit ameliorating effects on BPH. Further, my thesis research was focused on the androgen signaling inhibitory and anti-inflammatory effect of HX109 to unravel underlying mechanism of HX109.

First, the effects of HX109 on testosterone propionate(TP)-induced prostate hyperplasia rat model were tested. Oral administration of HX109 ameliorated prostate enlargement and histological change induced by TP. In LNCaP cells, a human prostate epithelial cell line, HX109 repressed androgen receptor (AR) -mediated cell proliferation but not epidermal growth factor (EGF)-mediated cell proliferation. In addition, HX109 suppressed the induction of AR target genes at the transcriptional level without affecting the translocation or expression of AR. Such effects of HX109 on AR signaling were mediated through the control of activating transcriptional factor 3 (ATF3) expression and phosphorylation of Calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ), as evidenced by data from experiments involving ATF3-specific siRNA and CaMKKβ inhibitor, respectively. These results indicated that HX109 might ameliorate symptoms of BPH via suppression of AR signaling.

Next, the effects of HX109 on inflammation, which is considered an important causative factor in BPH pathogenesis were examined using THP-1 macrophage cell line. In the co-culture system involving THP-1 macrophage and RWPE-1 prostate epithelial cell line, HX109 inhibited macrophage-induced cell

proliferation, migration and epithelial-mesenchymal transition (EMT). These inhibition is mediated by repressing the expression of CCL4 and the phosphorylation of STAT3. Furthermore, HX109 inhibited the expression of inflammatory cytokines and the phosphorylation of p65 NF-κB in a concentration dependent manner. These results suggested that HX109 could regulate macrophage activation and its crosstalk with prostate cells, thereby inhibiting BPH.

In conclusion, HX109 showed therapeutic effects on TP-induced prostate hyperplasia rat model. These effects might be mediated by suppressing AR signaling via upregulation of ATF3 and CaMKKβ or inhibiting macrophage crosstalk with prostate cells and its activation. Taken together with previous finding, HX109 may be developed as a safe and effective agent for the treatment of BPH.

Keywords: Benign prostatic hyperplasia(BPH), botanical drug, HX109, Testosterone, Androgen receptor(AR), macrophage, inflammation

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ABBREVIATIONS

AR	Androgen receptor
ARE	Androgen response element
ATF3	Activating transcriptional factor 3
BPH	Benign prostatic hyperplasia
CaMKKβ	Calcium/calmodulin-dependent protein kinase kinase β
CCL	CC-motif ligand
CREB	cAMP response element-binding protein
DHT	dihydrotestosterone
EGF	Epidermal growth factor
EtOH	Ethanol
FBS	Fetal bovine serum
GTA	General transcription apparatus
H&E	Hematoxylin & Eosin
HSP	Heat-shock proteins
IKK	IκB kinase
IL	Interleukin
LUTS	Lower urinary tract symptoms

NF-κB	Nuclear factor kappa-chain-enhancer of activated B cells
PCa	Prostate cancer
PSA	Prostate specific antigen
siRNA	Small interfering RNA
STAT3	Signal transducer and activator of transcription 3
TAK1	TGF-β activated kinase 1
TKM	Traditional Korean medicine
TLR4	Toll-Like-Receptor 4
TNFα	Tumor necrosis factor α
TP	Testosterone propionate
5ARI	5α -reductase inhibitors

Chapter I

Introduction

1. Benign Prostatic Hyperplasia(BPH)

1.1. Background

Benign prostatic hyperplasia (BPH) is one of the most common chronic diseases in the aged male population throughout the world. A normal prostate has a volume of 15-30 ml and a prostate larger than 30 ml is considered to be 'enlarged,' but there are no strict criteria [1]. BPH is characterized by a histological change in prostate architecture and variable growth of prostate size. The proliferation of the prostate's stromal and epithelial cells in the transitional zone surrounding the urethra is a notable characteristic of the disease (Figure 1). Increased prostate size tightens the urethra and induces lower urinary tract symptoms (LUTS) such as nocturia, dysuria, and bladder obstruction [2, 3].

It is reported that 50% of men over the age of 50 have enlarged prostates, with the incidence increasing with age and reaching 90% in men over 80 [4, 5]. As men age, the likelihood of developing associated LUTS increases in a linear manner [6](Fig 1). Since there is no strong relation between prostate size and symptoms, it is common to diagnose BPH based on symptoms rather than prostate size [7]. The prevalence of BPH varies by country and race, but it is clear that it is an age-related disease [8]. Although the worldwide prevalence of BPH is high, the etiology of the disease has yet to be fully elucidated. The progression of BPH

involves various factors including aging, hormonal changes, metabolic syndrome, oxidative stress, and inflammation [9-11]. Among these, androgen dysregulation is

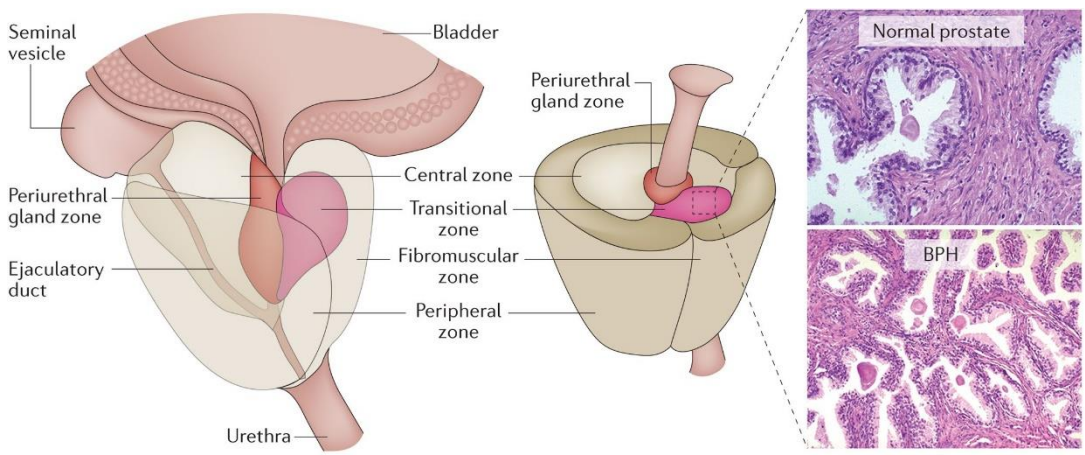


Figure 1. Schematic of prostate structure and BPH. Prostate has four regions: the non-glandular anterior fibromuscular zone, glandular peripheral zone, central zone and transitional zone. The urethra penetrates the periurethral gland zone, adjacent to the transition zone where BPH develops. Histological slides of both a normal prostate and BPH tissue are included. The BPH section shows proliferation of the glandular epithelial cells. (Taken from Chungtai, B. et al, *Nature review disease primer*, 2016)

particularly important, as it can induce prostate cell proliferation in both epithelial and stromal prostate cells.

BPH is often compared to prostate cancer (PCa) because both can enlarge the prostate gland and cause symptoms, like trouble urinating. The main difference between BPH and PCa lies in the cancerous nature of cell proliferation. In addition, most LUTS symptoms are caused by BPH, while only a small proportion of men have LUTS that are directly attributable to prostate cancer [12]. Anatomically, BPH arises from the transition zone of the prostate gland, while PCa is an adenocarcinoma, which classically arises from the epithelial cells located in the peripheral zone of the prostate gland[13]. BPH and PCa are linked at molecular and cellular level suggesting that these prostatic diseases have common pathobiological driving factors, but BPH may not make PCa more likely to occur [14, 15].

1.2. Role of androgen in BPH pathobiology

The androgen receptor (AR) is a member of the steroid receptor superfamily and is composed of three major domains: an N-terminal transcriptional activation domain, a central DNA-binding domain, and a C-terminal steroid-binding domain [16]. Once the AR binds to the androgens, heat-shock proteins(HSP) dissociate, and there is a conformational change in the AR for dimerization (Figure 2). The AR then interacts with coactivators and binds to a specific DNA site, the androgen

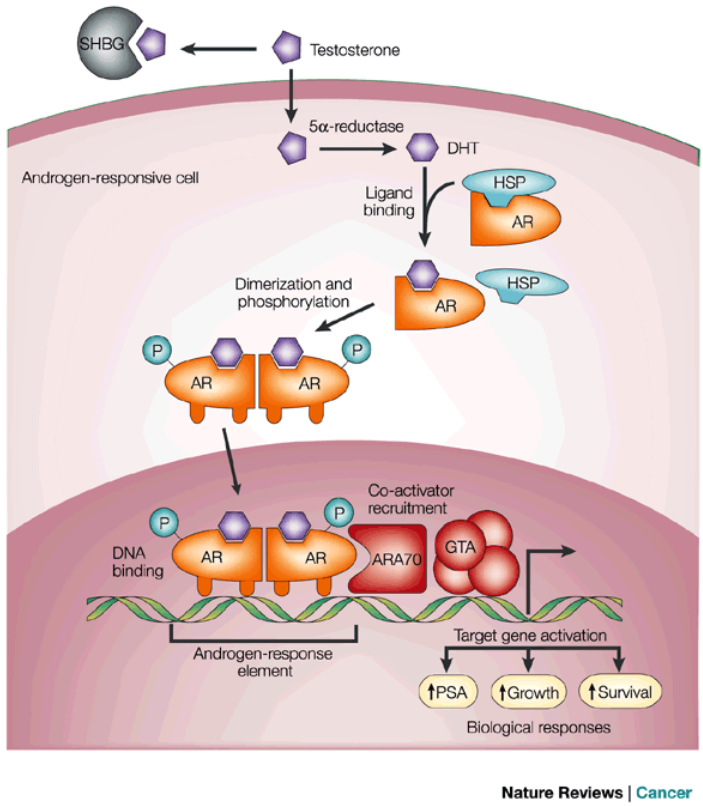


Figure 2. Androgen signaling pathway. Testosterone binds to sex-hormone-binding globulin (SHBG) and exchange with free testosterone. Testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase. Binding of DHT to the androgen receptor (AR) induces dissociation from heat-shock proteins (HSPs) and receptor phosphorylation. The AR dimerizes and can bind to androgen-response elements in the promoter regions of target genes. Co-activators or co-repressors also bind to the AR complex, regulating its interaction with the general transcription apparatus (GTA). (Taken from Feldman, B.J et al and Feldman, D., *Nature reviews cancer*, 2001)

response element (ARE), in the promoter of androgen-responsive genes to the activate transcription of these genes.

Among other causes, testicular androgens are required in the prostate for the development of BPH. In the prostate, testosterone is converted to dihydrotestosterone (DHT) by 5-reductase which is bound to the nuclear membrane. Both DHT and testosterone bind to the androgen receptor and promote protein synthesis involved in the cellular growth of prostate cells [17]. The serum concentration of testosterone and DHT may change with age, with reduced serum testosterone in healthy older men relative to that in healthy younger men. In contrast, DHT levels are elevated with aging, and serum DHT in BPH patients is significantly higher than that in unaffected men at a similar age[18, 19]. Also, castrated men or men who are affected by androgen impairment or production do not develop BPH. However, since there is no correlation between the concentration of androgens and prostate size, androgens are required for BPH development, but do not cause BPH [20].

1.3. Inflammation in BPH pathobiology

In the last few years, the role of prostatic inflammation as a key factor of prostate enlargement and BPH progression has become clearer. It has been hypothesized that various growth factors and cytokines involved in the inflammatory process and interaction with prostate cells are involved in BPH

development. One hypothesis is that infective etiology triggers the development of BPH. Several studies have reported the presence of heterogeneous bacterial and viral strain in BPH specimens [21-24]. It has also been shown that TLR agonists can induce the production of pro-inflammatory cytokines and chemokines by BPH stromal cells, and recruit infiltrating cells and promote prostate cell growth [25].

Different sources of prostatic inflammation have been proposed, including dietary factors, hormones, autoimmune responses and urine reflux [26]. The persistence of one or a combination of two or more of these stimuli might lead to chronic prostate inflammation. The chronic inflammatory condition may contribute to tissue injury, recruiting inflammatory infiltrates, activating cytokines release and increasing the concentration of growth factors[22, 27]. These growth factors and cytokines released from infiltrates (such as T cells, B cells and macrophage) promote the proliferation and development of BPH [28]. In addition, several clinical studies have reported that chronic inflammation contributes to the clinical progression of BPH-related symptoms [29-33].

1.4. Therapeutic approaches and drug market

As the world population ages, the incidence and prevalence of BPH have rapidly increased [34]. Although BPH is rarely fatal, its symptoms greatly reduce the quality of life. Various factors affect the progression of BPH, but therapy mainly targets androgen signaling to suppress prostate proliferation, or suppress

symptoms through relaxation of the smooth muscle (Table 1). The most frequently prescribed drugs for BPH are alpha blockers and 5α -reductase inhibitors (5ARIs) [35-37]. Alpha-blockers are antagonists of alpha receptor, a type of norepinephrine receptor, which block the receptors, preventing norepinephrine from working, and the smooth muscle relaxes the urine into the urethra. Although alpha blockers are widely used as an initial therapy for improving urine flow, they do not prevent the enlargement of the prostate gland and present side effects like hypotension [38]. 5-ARI is represented by finasteride targeting 5α -reductase type2 and dutasteride targeting both 5α –reductase type1 and type2. Both prevents prostatic enlargement by blocking the conversion of testosterone to DHT. 5ARIs are more effective and have a longer lasting effect than alpha-blockers, but have been reported to produce side effects such as the loss of libido and ability to ejaculate, and impotence [39, 40]. As such, there is a strong need for the development of safer and more efficacious agents for BPH.

2. Botanical formulation HX109

Since BPH is a chronic disease caused by multiple factors, plants may provide an interesting source for developing therapeutic agents [41]. There are several advantages to using botanical sources as therapeutic agents for BPH. First, many plants contain multiple components, so they can potentially interact with multiple cellular targets. Second, since many plants have traditionally been used as

Class	MOA	DRUG(Brand)	Daily Dose	Adverse Effects	30-Day AWP ($)[a]
Alpha-blockers	Relax tension in the prostate smooth muscle by targeting alpha-receptor	Alfuzosin (Uroxatral) Doxazosin (Cardura) Tamsulosin (Flomax) Terazosin (Hytrin) Silodosin (Rapaflo)	10 mg 1-8 mg 0.4-0.8 mg 1-10 mg 8 mg	Erectile dysfunction, abnormal ejaculation, dizziness, headache, orthostatic hypotension, fatigue, infection, polydipsia	126.41 40.46 126.41 48.11 249.47
5ARIs	Block the growth of prostate cells by targeting 5α-reductase enzyme and decreasing concentration of DHT	Dutasteride (Avodart) Finateride (Proscar)	0.5 mg 5 mg	Libido impairment, abnormal ejaculation, erectile dysfunction, mastalgia, gynecomastia	181.50 98.80
MRAs	Decrease bladder smooth muscle cell contractions by inhibiting muscarinic receptors	Fesoterodine (Toviaz) Tolterodine (Detrol LA) Tolterodine (Detrol)	4-8 mg 4 mg 1-2 mg twice daily	Xerostomia (dry mouth)	297.36 365.69 198.55
Combination product	Combined MOA from 5ARI and alpha-blocker (see above)	Dutasteride-tamsulosin (Jalyn)	0.5 mg/0.4 mg		181.52

Table 1. Pharmacological treatment option for BPH.

[a]Based on the least expensive available formulation (brand or generic) pricing for the regular-release formulation of initial dosing from Lexicomp, calculated to 30-day supply based on per unit pricing. 5ARIs, 5α-reductase inhibitors; AWP, average wholesale price; DHT, dihydrotestosterone; LA, long activating; MOA, mechanism of action; MRA, muscarinic receptor antagonist; (Taken from Yunou Wu et al, U.S. Pharmacist, 2016).

human medicines, historical evidence of their safety and efficacy is readily available [42]. In traditional Korean medicine(TKM), various plants sources are often used in mixture form, generally to strengthen therapeutic efficacy. Mixing plants is especially beneficial for treating chronic or complex diseases involving many different factors.

HX109 is an ethanol extract prepared from three plants: *Taraxacum officinale*, *Cuscuta australis*, and *Nelumbo nucifera*. These plants were selected on the basis of previous publications describing their activities and functions, and information available from traditional phytomedical practices in Asia. *Taraxacum officinale* has been widely used to treat urinary and renal diseases because of its diuretic, choleretic, anti-inflammatory, and anti-carcinogenic effects [43]. It has also been reported to inhibit prostate cancer cell proliferation and counteract inflammation [44, 45]. *Cuscuta australis* has been used as a tonic to treat urinary complaints, such as frequent urination and involuntary ejaculation [46]. The aqueous extract of *Nelumbo nucifera* has been reported to have antioxidant and anti-steroid properties that may inhibit androgen signaling [47-49]. Therefore, we hypothesized that the combination of these three plants might effectively address BPH symptoms.

3. Overview of thesis research

BPH is a chronic disease that occurs mainly in the elderly, and there is a need for the development of safe, effective, and long-lasting therapeutic agents. I developed HX109, a botanical formulation prepared from three plants: *Taraxacum officinale, Cuscuta australis, and Nelumbo nucifera*, to test its therapeutic effect on BPH. In this thesis, I initially tested the effects of HX109 on the rat BPH model and showed that oral administration of HX109 could suppress prostate weight gain and histological changes. Based on these observations, I further investigated the effects of HX109 on androgen signaling and inflammation, which play important roles in BPH pathobiology. Since androgen signaling plays the most important role in the development of BPH, the effects of HX109 on androgen signaling were studied in the prostate epithelial cell line. Then, the signaling molecules affected by treatment of HX109 were screened in order to better understand the underlying mechanism by which HX109 regulates androgen signaling. Finally, the effects of HX109 on inflammation were studied using a co-culture system involving macrophage-prostate epithelial cells. The effects of HX109 on proliferation, migration, and EMT of prostate epithelial cells increased by co-culture with macrophages and its underlying molecular mechanism were investigated. In addition, the anti-inflammatory effects of HX109 were tested in the macrophage cell line. In summary, my thesis focuses on identifying the therapeutic effects of HX109 on BPH *in vivo* and understanding how HX109 works in androgen signaling and inflammation situations.

Chapter II

Materials and Methods

1. Cell culture and reagents

1.1. preparation of HX109

HX109 is an ethanol extract prepared from three plants: *Taraxacum officinale*, *Cuscuta australis*, and *Nelumbo nucifera*. All plants used in the preparation of HX109 were purchased from Humanherb (Gyeongsan, Korea) and authenticated through the plant identification services of Plant DNA Bank in Korea (PDBK, Seoul, Korea) using their genome sequences. HX109 was prepared by mixing Taraxacum officinale, Cuscuta australis, and Nelumbo nucifera at a ratio of 2:1:1. The combination of plants (total dry weight, 60 g) was extracted with 600 mL of 25% EtOH at 20 ℃ for 8 h. The extract was filtered with 10-μm cartridge paper and concentrated using a rotary evaporator (Eyela, Tokyo, Japan), followed by a freeze-drying process. This process generally produced approximately 8.5 g of brown powder with a yield of about 14%. The voucher specimens used in this study had been deposited in the herbarium of ViroMed Co., Ltd. (Seoul, Korea).

1.2. High-performance liquid chromatography (HPLC) analysis

High-performance liquid chromatography analysis was employed to validate the quality of HX109. Reference standards for chicoric acid, maltol, dihydrophaseic acid, and isoschaftoside were used for qualitative and quantitative analyses of HX109. Analytical samples of HX109 were studied by HPLC-PDA (Waters, USA)

with Capcell PAK C18 MG column (4.6mm x 250mm, 5μm, Shiseido, Japan). The mobile phase was water (0.05% trifluoroacetic acid) for solvent A and acetonitrile (0.01% trifluoroaceetic acid) for solvent B. The mobile phase gradient was 5 - 27% B (0–10 min), 27 – 35% B (10–25 min), 35 - 100% B (25–30 min); the flow rate was 1.0 ml/min, and the injection volume was 5 μl. The sample concentration was 20 mg/ml.

1.3. chemical reagents

EGF was purchased from R&D systems (Minneaplis, MN) and used at 10 ng/ml for experiment. STO-609 (a CaMKKβ inhibitor, Tocris Bioscience, MO) was used at 30 μM and BAPTA-AM (a calcium chelator, Sigma-Aldrich; St Louise, MO) was used at 20 μM for the experiment. Lipopolysaccharides (LPS) from Escherichia coli O111:B4 were purchased from Sigma-Aldrich and used at 100 ng/ml for macrophage activation.

1.4. LNCaP cell culture

LNCaP human prostate cancer cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in RPMI-1640 medium (Corning, Corning, NY) supplemented with a 10% heat-inactivated fetal bovine serum (FBS; Corning), HEPES (10 mM), penicillin and streptomycin

in a humidified 5% CO_2 atmosphere at 37 ℃. To examine the effects of TP, cells were cultured in phenol red-free RPMI1640 containing 5% charcoal stripped serum (CSS; TCB, Long Beach, CA) for 24 h, and 100 nM TP was then added to the medium.

1.5. RWPE-1 cell culture

RWPE-1 cells (human prostate epithelial cell line) were purchased from ATCC. Cells were cultured in Keratinocyte-serum free medium (KSFM; Thermo Fisher Scientific, Waltham, MA) supplemented with bovine pituitary extract (BPE) and EGF in a humidified 5% CO_2 atmosphere at 37 ℃.

1.6. THP-1 cell culture

THP-1 cells (human acute monocytic leukemia cell line) were purchased from ATCC. Cells were maintained in RPMI1640 (Corning) containing 10% heat-inactivated FBS (Corning), HEPES (10 mM), penicillin and streptomycin in a humidified 5% CO_2 atmosphere at 37 ℃. THP-1 cells were differentiated into macrophages with 60 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich) for 48h. Then the plates were washed with PBS and incubated with normal RPMI1640 with 10% FBS medium for 48 h, and later used for further experiments.

1.7. Co-culture experiments

Co-culture experiments were performed using 24-well transwell inserts (0.4um pore; Corning). RWPE-1 cells (10^4 cells/well) were seeded in 24-well transwell plates and insert wells including THP-1 cells (10^4 cells/well) were put into each cell seeded-well and cultured with or without HX109 treatment. Cells were harvested for 48 h and WST-1 assay or RNA isolation were performed. For protein preparation, cells were harvested for 24 h and total proteins were prepared from RWPE-1 cells.

1.7. Raw264.7 cell culture

Raw264.7 cells were purchased from ATCC. Cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with a 10% heat-inactivated FBS (Corning) and penicillin and streptomycin in a humidified 5% CO_2 atmosphere at 37 ℃.

2. Molecular techniques

2.1. Measurement of cell proliferation (WST-1 assay)

For LNCaP cells, cells were seeded at a density of 5 x 10^4 cells/well in 24-well CellBIND (Corning) plates (n=3). After androgen starvation, cells were incubated with TP and HX109 at designated concentration for designated time periods. After incubation, cell proliferation was examined by CellVia WST-1 assay according to the manufacturer's protocol (Young In Frontier, Seoul, Korea).

2.2. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

To determine the RNA level in LNCaP cells, cells were seeded at a density of 1 x 10^5 cells/well in 12-well CellBIND plates (n=3). After androgen starvation, cells were incubated with TP and HX109 at designated concentration for designated time periods. To determine the RNA level in RWPE-1 cells, cells were prepared after co-culture with THP-1 for 24 h or 48 h. To determine the RNA level in THP-1 cells or Raw264.7cells, cells were treated with 100 ng/ml LPS and various concentration of HX109 for 3 h.

Total RNAs were prepared from LNCaP cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One microgram of RNA was converted to cDNA using oligo dT primers (QIAGEN, Hilden, Germany) and Reverse Transcriptase XL (avian myeloblastosis virus [AMV]) (Takara, Kusatsu, Japan). Real-time quantitative RT-PCR was performed with SYBR Premix (Takara) and Thermal Cycler Dice Real Time System TP800

(Takara). PCR conditions were denaturation at 95 °C for 5 seconds, followed by annealing and extension at 60 °C for 30 seconds for 40 cycles. RNA levels were normalized by the level of GAPDH and the relative changes in gene expression were calculated as $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were described in Table 2.

2.3. Western blot analysis

To determine the protein level in LNCaP cells, cells were seeded at a density of 5 x 10^5 cells/plate in 60 mm cell culture dish or 2 x 10^5 cells/well in 6-well CellBIND plate. After treatment with TP and HX109, Cells were washed with cold PBS lysed with RIPA lysis buffer (Sigma-Aldrich) containing a protease inhibitor (Roche, Basel, Switzerland) and a phosphatase inhibitor (Roche). Equal amounts of protein were then separated by 10% SDS-polyacrylamide gel and electrophoretically transferred to PVDF membranes (Millipore, MA). The membranes were blocked with 5% BSA (Gibco, MA) in TBST (1 M Tris-HCl [pH 7.4], 0.9% NaCl, and 0.1% Tween 20) for 1 h and incubated with primary antibodies diluted in a 3% BSA blocking solution overnight at 4°C. Membranes were then treated with horseradish peroxidase (HRP) -conjugated anti-mouse or anti-rabbit IgG (1: 100,000; Sigma-Aldrich) for 1 h, and protein bands were visualized with an ECL (Millipore) and X-Omat film (Kodak, Rochester, NY).

To determine the protein level in RWPE-1 cells, cells were prepared after

Target gene	Primer sequence (5'->3')	
	Forward	**Reverse**
hKLK3(PSA)	GTGTGTGGACCTCCATGTTATT	CCACTCACCTTTCCCCTCAAG
hATF3	AAGAACGAGAAGCAGCATTTGA	TTCTGAGCCCGGACAATACAC
hKLK2	ATGTGTGCTAGAGCTTACTC	AAGTGGACCCCCAGAATCAC
hTMPRSS2	GGACAGTGTGCACCTCAAAGAC	TCCCACGAGGAAGGTCCC
hDHCR24	GAGGCAGCTGGAGAAGTTTG	CTTGTGGTACAAGGAGCCATC
hNKX3.1	CCTCCCTGGTCTCCGTGTA	TGTCACCTGAGCTGGCATTAC
hE-cadherin	CGAGAGCTACAC GTTCACGG	GTGTCGAGGGAAAAATAGGCTG
hN-cadherin	GTTTGATGGAGGTCTCCTAACAC	ACGTTTAACACGTTGGAAATGTG
Snail	GAGGCGGTGGCAGACTAGAGT	CGGGCCCCCAGAATAGTTC
hCCL3	AGTTCTCTGCATCACTTGCTG	CGGCTTCGCTTGGTTAGGAA
hCCL4	CTGTGCTGATGATCCCAGTGAATC	TCAGTTCAGTTCCAGGTCATACA
hCCL5	ATCCTCATTGCACTGCCCTC	GCCACTGGTGTAGAAATACTCC
hIL-6	CGGGAACGAAAGAGAAGCTCTA	CGCTTGTGGAGAAGGAGTTCA
hTNFα	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
hGAPDH	CCCCTTCATTGACCTCAACT	ATGACCTTGCCCACAGCCTT
mIL-6	CCTCTGGTCTTCTGGAGTACC	ACTCCTTCTGTGACTCCAGC
mTNFα	ATGAGCACAGAAAGCATGA	AGTAGACAGAAGAGCGTGGT
mGAPDH	CTGGAAAGCTGTGGCGTGAT	CCAGGCGGCACGTCAGATCC

Table 2. Sequences of primers used for qRT-PCR analysis.

co-culture with THP-1 for 24 h. The identical Western blot procedures were performed to detect the protein level of RWPE-1 cells.

To determine the protein level in THP-1 cells, cells were treated with 100 ng/ml LPS and various concentration of HX109 for 30 min. The identical Western blot procedures were performed to detect the protein level of RWPE-1 cells.

The primary antibodies used in this study were as follows: PSA (ab76113), CAMKKβ (ab168818), GAPDH (ab9485) (1:1000; abcam, Cambridge, MA), AR (06-680; Millipore), Histone H3 (#4499), ATF3 (#33593), phospho -CAMKKβ (#12818), phospho-STAT3 (#9145), STAT3 (#4904), phospho-TAK1 (#9339), TAK1 (#5206), phospho-IKK (#2694), IKK (#2684), phospho-p65 (#3039), p65 (#8242), IkBα (#9242) (1:1000; Cell Signaling Technology, Danvers, MA)

2.4. Enzyme-linked immunosorbent assay (ELISA)

To measure DHT and PSA levels, ELISA kits specific to DHT (ALPCO Diagnostics, Salem, NH, USA) and PSA (Cusabio, TX) were used according to the manufacturer's instructions. When in vivo samples were prepared, sera were used to detect DHT, and levels were expressed as pg/ml. Prostate samples were homogenized using T-PER tissue protein lysis Buffer (Thermo Fisher Scientific) containing a protease inhibitor (Roche) and a phosphatase inhibitor (Roche). After preparation, samples were centrifuged at 12,000 rpm for 10 min at 4 °C and the

supernatants were used to detect DHT and PSA. Values from prostate proteins were normalized by total proteins and expressed as pg/mg protein.

To measure inflammatory cytokines in THP-1 cells and Raw264.7 cells, the production of Human TNFα, IL-6, mouse TNFα, IL-6 was evaluated by ELISA. Briefly, cells were treated with 100 ng/ml LPS and incubated for 24 h. Human TNFα, IL-6, mouse TNFα, IL-6 (R&D systems) in cell culture supernatants were measured using commercially available ELISA kits according to the manufacturer's instructions.

2.5. Luciferase reporter plasmid assay

An inducible AREs-responsive luciferase reporter assay kit was purchased from QIAGEN (Valencia, CA), and the assay was performed as described previously [14]. LNCaP cells were briefly transfected with ARE-reporter plasmid or negative control plasmid using lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were treated with TP (100 nM) and various concentrations of HX109 for 18 h. Cell lysates were prepared, and a luciferase activity assay was performed using the dual luciferase reporter assay system (Promega, Madison, WI) and microplate luminometer (MicroLumat Plus LB96V, Berthold, Germany) according to the manufacturer's protocol. The data are shown as the ratio of Firefly luciferase activity to Renilla luciferase activity (Fluc/Rluc).

2.6. Extraction of nuclear and cytoplasmic fractions

Fractionation and extraction of nuclear and cytoplasmic proteins from LNCaP cells treated with TP and HX109 for 3 h were performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's protocol.

2.7. siRNA-mediated gene knockdown

For siRNA-mediated gene knockdown of ATF3, 1 x 10^5 cells (n=3) were seeded in 12-well CellBIND plate. The siRNA specific to ATF3 and scrambled siRNA (Thermo Fisher Scientific) were transfected into LNCaP cells using RNAiMAX (Invitrogen) according to the manufacturer's instructions. 24 h after the siRNA mediated knockdown of ATF3, cells were briefly treated with TP and HX109, and subjected to further analysis. Knock-down efficiency was evaluated using an antibody against ATF3.

2.8. Calcium assay

For the calcium assay, LNCaP cells were plated at 5 x 10^4 cells per well in a 24-well CellBIND plate containing phenol red-free RPMI with 10% FBS. Twenty-

four hours later, cells were treated with TP and 1 mg/ml HX109. After 1 and 5 min, cells were washed with PBS and lysed with PBS containing 0.5% Triton X. The calcium level of cell lysates was measured using a calcium assay kit (Sigma-Aldrich) according to the manufacturer's protocol.

2.9. Cell migration assay

Cell migration assay was performed using 24-well transwell inserts (8um pore; Corning) according to the manufacturer's instructions. RWPE-1 cells (10^5 cells/well) were seeded in the upper chamber of transwell plates and THP-1 cells (10^5 cells /well) or control medium was added to the lower chamber. Cells were incubated for 24 h with or without HX109 treatment. The cells migrated to the lower part of the membrane were stained with 0.2% crystal violet and counted in six random fields.

2.10. Statistical analysis

All values are presented as mean ± SEM from three independent experiments. Each experiment was repeated at least three times, independently. Statistical significance was determined using unpaired Student's t test or one-way ANOVA with Turkey correction, provided by the GraphPad Prism 7 software (GraphPad, San Diego, CA). Data were considered statistically significant if the p-

value was less than 0.05.

3. Animal Studies

3.1. Experimental Animals

Ten-week old male Sprague Dawley (SD) rats weighing 330 ± 20 g were obtained from Orient Bio (Seongnam, Korea) for animal studies. Animals were housed under controlled environmental conditions: constant temperature (25 ± 2 °C), humidity (60 ± 10%), and a 12 h light/ dark cycle. All experiments were performed according to the guidelines set by the International Animal Care and Use Committee at Seoul National University.

3.2. TP-induced BPH rat model

Rats were acclimatized for 1 week, followed by bilateral orchiectomies to prevent the influence of endogenous testosterone. After 1 week, rats were divided into 5 groups: NC, BPH, HX200, HX300, and Fina (n=5 per group). Prostatic hyperplasia was induced in 4 groups (BPH, HX200, HX300, Fina) by subcutaneous injection of 3 mg/kg of testosterone propionate(TP) (TCI, Tokyo, Japan) dissolved in cottonseed oil (Sigma-Aldrich) every three days. The CAS group received only cottonseed oil in order to provide similar subcutaneous injection conditions in all

groups. During the induction of prostate hyperplasia, rats orally received respective reagents on a daily basis for 4 weeks. The HX200 group and HX300 group were orally administrated HX109 200 mg/kg or HX109 300 mg/kg. The Fina group was orally administrated 5 mg/kg of Finasteride as a positive control. The CAS group and BPH group were orally administrated TDW as a vehicle. Body weight was measured once a week during the experiment. After 4 weeks, rats were sacrificed, and prostates were immediately removed and weighed.

3.3. Histological analysis

Prostates were fixed in 10% normalized buffered formalin (Sigma-Aldrich) and embedded in the paraffin block. Then, 6 μm paraffin sections of the prostate were stained with H&E to analyze acinar areas. The size of each acinus was measured by ImageJ software (NIH, MD).

Chapter III

Therapeutic effects of HX109 in TP-induced BPH rat model and Regulation of AR signaling by HX109 through Ca2+/CaMKKβ and ATF3

1. Background

Benign prostatic hyperplasia (BPH) is a common disease in the elderly male population throughout the world. Among other factors, androgen dysregulation has been known to play major roles in its pathogenesis. In the prostate, testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase. Both DHT and testosterone bind to androgen receptor (AR) and promote protein synthesis involved in the cellular growth of prostate cells [17]. Since androgen/AR signaling plays an important role in the pathogenesis of BPH, many therapeutic agents are being developed to target this pathway.

AR is a receptor of androgen normally present in the cytoplasm, which translocates into the nucleus upon binding the androgen. In the nucleus, AR regulates gene expression by binding to androgen receptor response elements (AREs) located in the promoter and enhancer region of various target genes [50]. Androgen-mediated gene expression is affected by a number of different factors. Activating transcriptional factor 3 (ATF3), known as a common stress response mediator, is a member of the ATF/cAMP response element-binding protein (CREB) family of transcriptional factor [51]. ATF3 represses AR transactivation by binding to the transcription domain of the AR. It was reported that the loss of ATF3 results in increased prostate cell proliferation as well as the transcription of androgen-target genes [52]. Calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ), which is activated by Ca2+/calmodulin, is also known to influence AR

signaling. It has been reported that CaMKKβ overexpression inhibits AR-mediated gene expression, whereas the knockdown of CaMKKβ enhances AR-signaling and proliferation [53].

2. Results

2.1. Quality of HX109 Is Monitored by HPLC Analysis and Cell-Based Bioassay

To establish batch-to-batch consistency of research grade HX109, one representative marker compound from each plant (chicoric acid for *Taraxacum platycarpum*, maltol for *Cuscuta australis*, and dihydrophaseic acid for *Nelumbo nucifera*) was used, based on previously published information [54-56]. In addition, isoschaftoside was used (Figure 3) as a qualitative marker of *Nelumbo nucifera* [57]. The marker compounds were analyzed using HPLC (Figure 4), and only the extracts containing these compounds within the set range (4.8±0.2 mg/g for chicoric acid, 0.3±0.1 mg/g for maltol, and 0.1±0.05 mg/g for dihydrophaseic acid) were used for this study (Table 3). The identifications of these marker compounds were further confirmed by mass spectrometer (Figure 5,6).

In addition, the quality of HX109 was biologically controlled using cell-based bioassays. LNCaP cells were treated with TP in the presence of various concentrations of HX109 and the effect on prostate specific antigen (PSA) secretions was determined to calculate the IC50 value (Figure 7). Only HX109

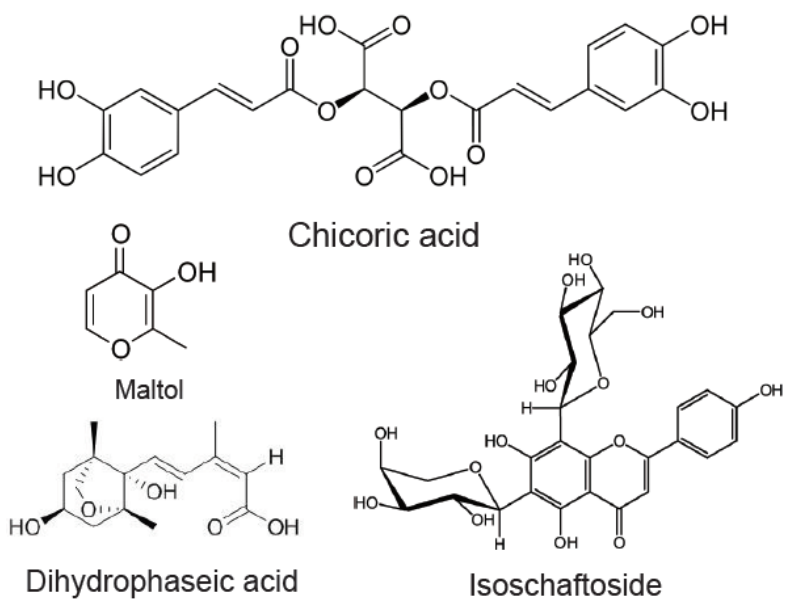


Figure 3. Molecular structure of marker compounds.

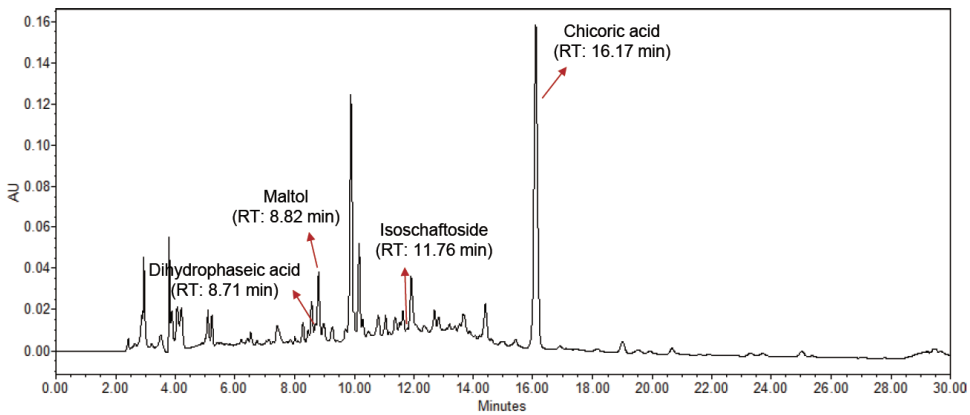


Figure 4. Representative HPLC chromatogram of HX109. HX109 was dissolved in 50% methanol at a concentration of 20 mg/ml and subjected to HPLC analysis. HPLC was performed as described in Materials and Methods. HPLC was performed as described in Material and Method. The marker compounds in HX109 were identified by comparison with the reference standard and the peaks of marker compounds are indicated by arrows. RT, retention time.

Batch No.	Chicoric acid (mg/g)	Maltol (mg/g)	Dihydrophaseic acid (mg/g)	Isoschaftoside
Batch 1	4.69	0.32	0.14	Detected
Batch 2	4.84	0.31	0.13	Detected
Batch 3	4.74	0.21	0.15	Detected

Table 3. Quantification of marker compounds in HX109

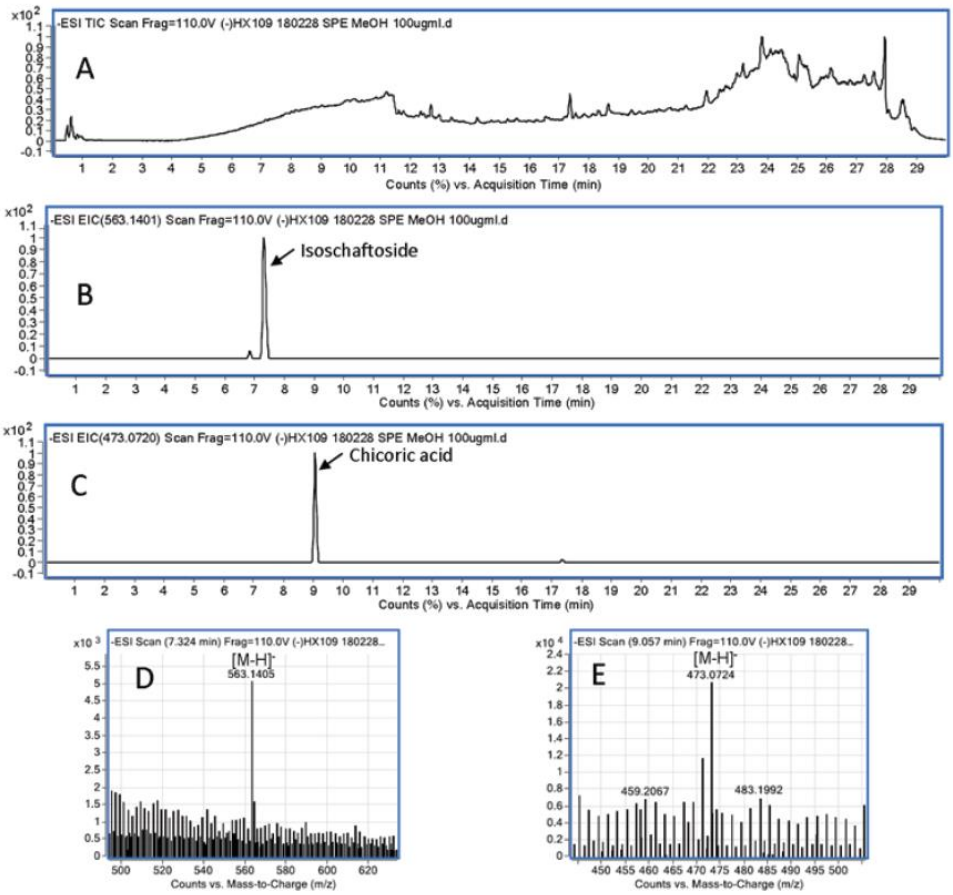


Figure 5. HPLC-ESI-Q-TOF-MS chromatogram of HX109 (negative ion mode). The theoretical MS of isoshcaftoside and chicoric acid are 563.1401 $[M-H]^-$ and 473.0720 $[M-H]^-$, respectively. (A) Total ion chromatogram of HX109. (B) Extracted ion chromatogram of HX109 at m/z 563.1401. (C) Extracted ion chromatogram of HX109 sample at m/z 473.0720. (D) and (E) MS spectrum of mass peak at 7.324 and 9.057 min. The mass spectrum of both peaks showed [M-H]- ion peaks at m/z 563. 1405 and 473.0724, respectively. The error rate was within 5 ppm.

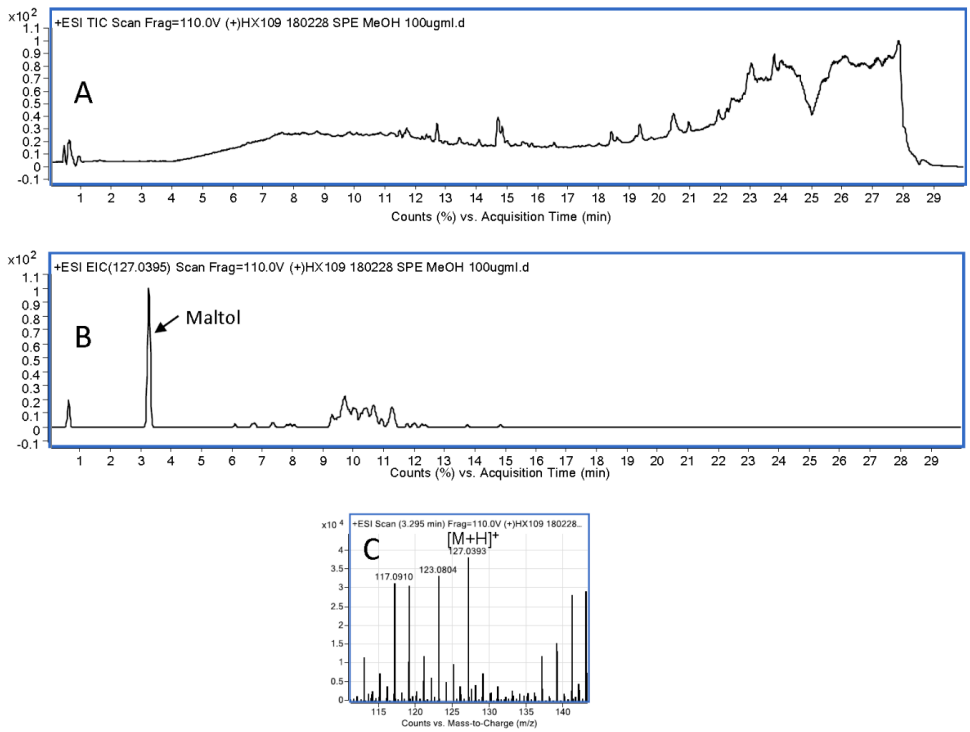
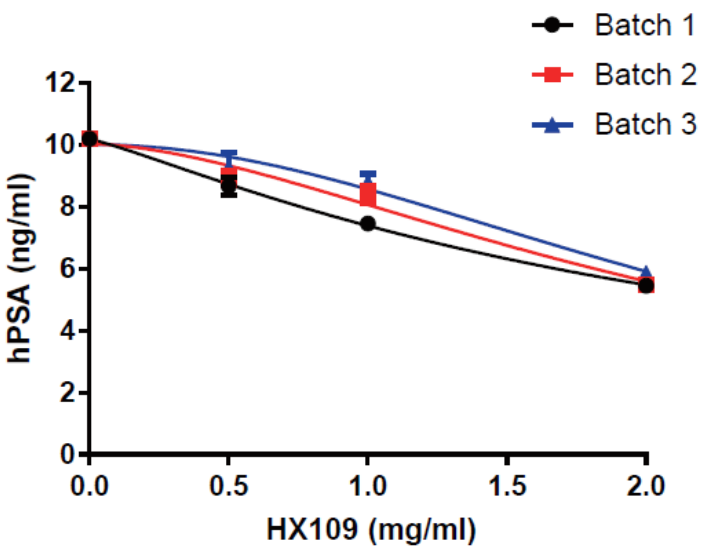


Figure 6. HPLC-ESI-Q-TOF-MS chromatogram of analytical sample for HX109 (positive ion mode). The theoretical MS of maltol is m/z 127.0395 $[M+H]^+$; (A) Total ion chromatogram of HX109 sample; (B) Extracted ion chromatogram of HX109 sample at m/z 127.0395; (C) MS spectrum of mass peak at 3.295 min. The error rate was within 5ppm.



Batch	IC50 (mg/ml)
1	2.273
2	2.294
3	2.395

Figure 7. Cell-based bioassay of HX109 in LNCaP cells. LNCaP cells were treated as described in Fig. 4A. The effects of different batches of HX109 on hPSA protein secretion were analyzed by ELISA after 24 h. Half maximal inhibitory concentration (IC50) values of three batches were calculated from the graph. Data are shown as mean ± S.E.M of three independent experiments.

preparations showing IC50 values between 2.2 to 2.4 mg/ml were used for the experiments.

2.2. HX109 Ameliorates TP-Induced Benign Prostatic Hyperplasia in Castrated Sprague Dawley Rat

To test the effects of HX109 on BPH, a testosterone propionate (TP)-induced BPH rat model was used [58]. Injection of TP every three days into castrated Sprague Dawley rats for 4 weeks (designated as the BPH group) caused a significant ($p < 0.001$) increase in prostate weight and index compared to the castration-only group (NC) (Table 4). In rats orally administrated with HX109 for 4 weeks, however, both prostate weight and index decreased in a dose-dependent manner. Prostate weight was reduced by 33% in rats receiving 300 mg/kg HX109 compared to the BPH control group, while finasteride, used as a positive control, decreased it by 43%. Body weight was not affected in all groups (Table 4).

H&E staining was performed to investigate the effect of HX109 on histological changes in the prostate. As shown in Figure 8, administration of TP increased the area of prostatic acinar 6-fold compared to the NC group, but the area size decreased by 35% following HX109 treatment (Figure 8B). The effect of HX109 on the protein levels of PSA, a frequently used marker of prostate enlargement, was also investigated. The PSA levels in the prostate were markedly higher in the BPH group than in the NC group. However,

Group	Body weight (g)		Prostate weight (g)	Prostate index (mg / 100 g body weight)	Growth inhibition (%)
	Initial	Final			
NC	356.93 ± 4.58	445.26 ± 5.42	0.0323 ± 0.0026	0.73 ± 0.063	
BPH	356.12 ± 5.79	436.29 ± 8.05	0.779 ± 0.025$^{\#\#\#\#}$	17.88 ± 0.64$^{\#\#\#\#}$	
HX109 200 mg/kg	356.56 ± 5.67	421.35 ± 10.15	0.686 ± 0.042	16.44 ± 1.28	8.4%
HX109 300mg/kg	356.44 ± 5.09	432.98 ± 8.60	0.528 ± 0.036****	12.17 ± 0.72***	33.29%
Fina	536.05 ± 8.29	429.99 ± 11.26	0.454 ± 0.018****	10.55 ± 0.15****	42.74%

Table 4. Effects of HX109 on body weight and prostate weight in TP-induced BPH rats. All data are mean ± standard error of the mean (S.E.M.). Growth inhibition = 100 - ((treated group-NC group)/(BPH group-NC group) x 100). NC : cottonseed oil subcutaneous injection(s.c.) + TDW orally treated(p.o.); BPH : TP 3 mg/kg s.c. + TDW p.o; HX109 200 mg/kg : TP s.c. + HX109 200 mg/kg p.o; HX109 300 mg/kg : TP s.c. + HX109 300 mg/kg p.o.; Fina : TP s.c. + Finasteride 10 mg/kg p.o.; $^{\#\#\#\#}p<0.0001$ vs NC group. ***$p<0.001$, ****$p<0.0001$ vs BPH group.

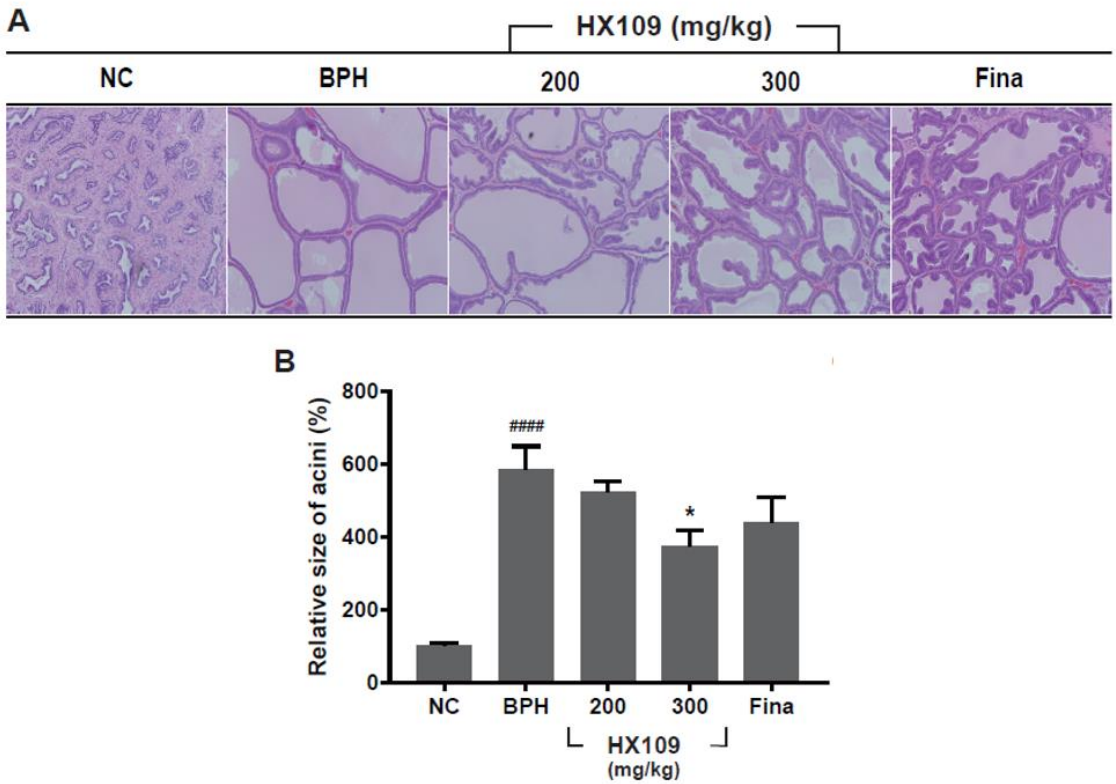


Figure 8. Effects of HX109 on prostate histology in BPH model. Castrated Sprague Dawley rats were injected with 3 mg/kg every three days and orally administrated with TDW (BPH) or HX109 200 mg/kg or HX109 300 mg/kg or Finasteride 5 mg/kg (Fina). Rats injected with vehicle were used as a negative control (NC) group. (A) Effects of HX109 on histological changes in prostate. After measuring the final prostate weight, rats were sacrificed and prostate tissues were fixed, sectioned, and stained with hematoxylin and eosin (H&E). (B) Relative size of acinal area. Values were calculated from three randomly captured pictures. $^{####}p<0.0001$ (one-way ANOVA) compared with the NC group, $*p<0.05$ (one-way ANOVA) compared with BPH group. All Data are shown as mean ± S.E.M

they were reduced in the HX109-treated group compared to the BPH group (Figure 9).

In the prostate, the major prostatic androgen is DHT, which is converted by 5-reductase from testosterone [59]. When DHT levels in the serum and prostate were measured, they were increased in the BPH group compared to the NC group, and HX109 treatment had little effect on DHT levels (Figure 10). These results indicated that 5-reductase might not be involved in the HX109-mediated amelioration of prostate enlargement.

2.3. HX109 Suppresses Androgen-Dependent Proliferation of LNCaP Cells

In the BPH rat model, the increase in prostate weight by TP is well known to be induced by prostate cell proliferation. Therefore, it was tested whether HX109 could control androgen-induced prostate cell proliferation. LNCaP cells were treated with various concentrations of HX109, with or without the addition of TP, and effects on cell proliferation were measured by WST-1 assay. As shown in Figure 11A, treatment with TP increased proliferation of LNCaP cells by about 56% compared to the vehicle group, but HX109 treatment inhibited TP-induced cell proliferation in a dose-dependent manner. At 2 mg/mL of HX109, TP-induced cell proliferation was inhibited by almost 50%. These effects were not due to cytotoxicity, as cell viability was not affected by HX109 (Figure 11B). In addition, HX109 treatment didn’t inhibit epidermal growth factor (EGF) induced

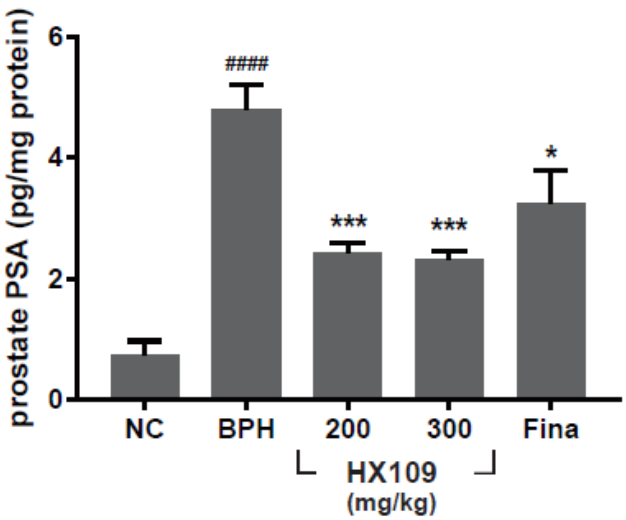


Figure 9. Effects of HX109 on prostate PSA levels. PSA levels of rat prostate were measured by ELISA. Values were normalized to total proteins. n=5 per group. ####p<0.0001 (one-way ANOVA) compared with the NC group, *p<0.05, ***p<0.001 (one-way ANOVA) compared with BPH group. All Data are shown as mean ± S.E.M.

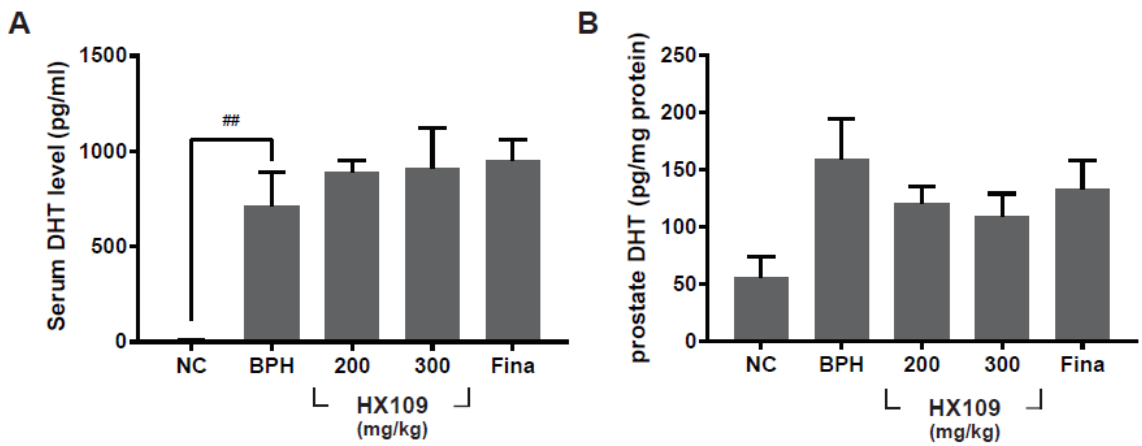


Figure 10. Effects of HX109 on serum and prostate DHT level. Castrated Sprague Dawley rats were injected with TP 3 mg/kg every three days and orally administrated with TDW (BPH) or HX109 200 mg/kg or HX109 300 mg/kg or Finasteride 5 mg/kg (Fina). Rats injected with vehicle were used as negative control (NC) group. After rats were sacrificed, the DHT levels of rat serum (A) or prostate (B) were measured by ELISA. $^{\#\#}p<0.01$ (one-way ANOVA) compared with the NC group. n=5 per group. All Data are shown as mean ± S.E.M.

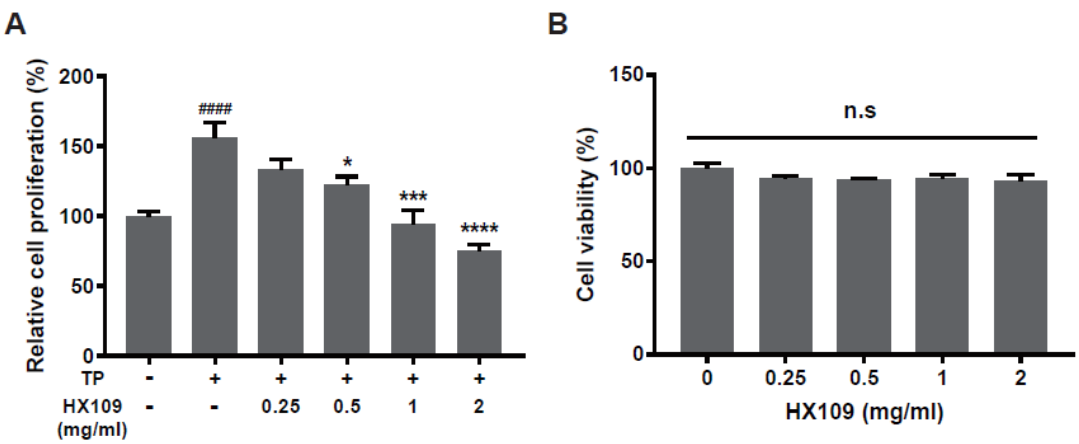


Figure 11. Effects of HX109 on androgen-dependent proliferation of LNCaP cells. LNCaP cells were plated in culture media for 24 h followed by androgen starvation using phenol red free RPMI1640 containing 10% charcoal-stripped serum for 24 h. After androgen starvation, LNCaP cells were treated with or without 100 nM TP and cultured in the presence of various concentrations of HX109 for 72 h. Cell proliferation was measured by WST-1 assay. (A) Effects of HX109 on the TP-induced proliferation of LNCaP cells. (B) Cytotoxicity effects of HX109. $^{\#\#\#\#}p<0.0001$ (one-way ANOVA) compared with control, $*p<0.05$, $***p<0.001$, $****p<0.0001$ (one-way ANOVA) compared with TP only. Values are normalized to control. All data are shown as mean ± S.E.M of three independent experiments.

proliferation (Figure 12). These data suggested that HX109 might inhibit the androgen-dependent proliferation of LNCaP cells.

2.4. HX109 Inhibits Androgen-Induced PSA Expression

Androgen-dependent proliferation is regulated by androgen/androgen receptor (AR) signaling. Since PSA is the main target of AR signaling, the effect of HX109 on this protein was investigated. In the absence of TP, LNCaP produced a small amount of PSA, but treatment with 100 nM TP elevated its level by 10-fold. When treated with HX109, PSA levels in the cell culture supernatant were decreased in a dose-dependent manner, maximally by 50% at 2 mg/mL (Figure 13A). Suppression of PSA expression by HX109 was also observed by western blot (Figure 13B).

To determine whether HX109 affects the production PSA at a transcriptional level, the RNA level of PSAs was measured by quantitative RT-PCR. TP-induced RNA expression of PSA was reduced by HX109 in a dose-dependent manner (Figure 14). At 2 mg/mL HX109, the RNA level of PSAs was lowered by 80% compared to TP only. To further verify the effects of HX109 on AR signaling, the RNA levels of other downstream target genes of AR—such as KLK2, TMPRSS2, DHCR24, and NKX3.1—were also measured. The expression of all target genes was highly downregulated when treated with HX109 (Figure 15). These results indicated that HX109 could effectively inhibit AR-mediated gene

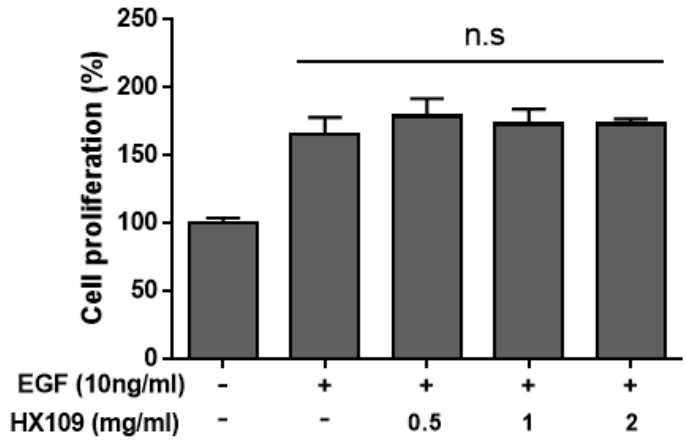


Figure 12. Effects of HX109 on EGF-dependent proliferation of LNCaP cells. LNCaP cells were plated in culture media for 24 h followed by androgen starvation using phenol red free RPMI1640 containing 10% charcoal-stripped serum for 24 h. After androgen starvation, LNCaP cells were treated with or without 10 ng/ml EGF and cultured in the presence of various concentrations of HX109 for 72 h. Cell proliferation was measured by WST-1 assay. n.s, not significant. All values are shown as mean ± S.E.M of three independent experiments.

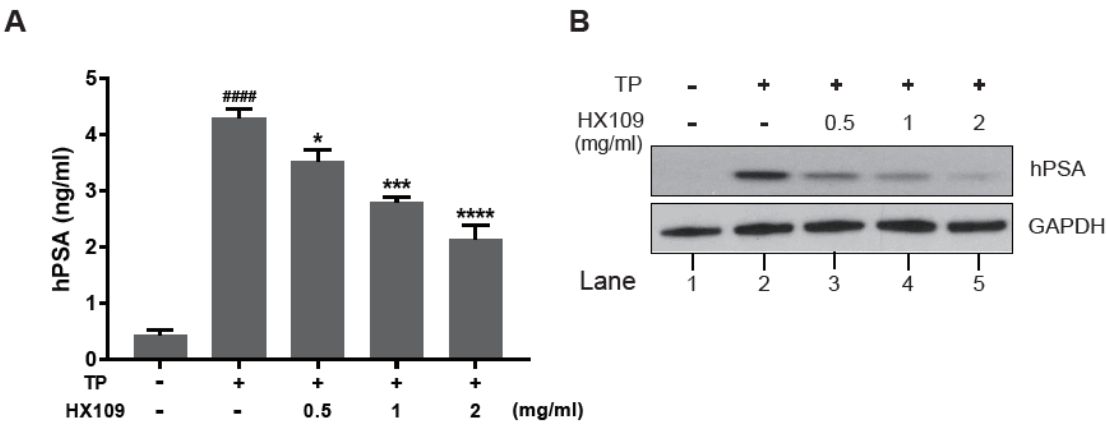


Figure 13. Effects of HX109 on androgen-induced PSA(KLK3) protein expression. LNCaP cells were treated as described in Figure 11. (A) Effects of HX109 on hPSA protein secretion were analyzed by ELISA after 24 h. ####p<0.0001 (one-way ANOVA) compared with control, *p<0.05, ***p<0.001, ****p<0.0001 (one-way ANOVA) compared with TP only. All values are shown as mean ± S.E.M of three independent experiments. (B) Effects of HX109 on hPSA protein expression. After 48 h, protein levels in cell lysate were analyzed by Western blot.

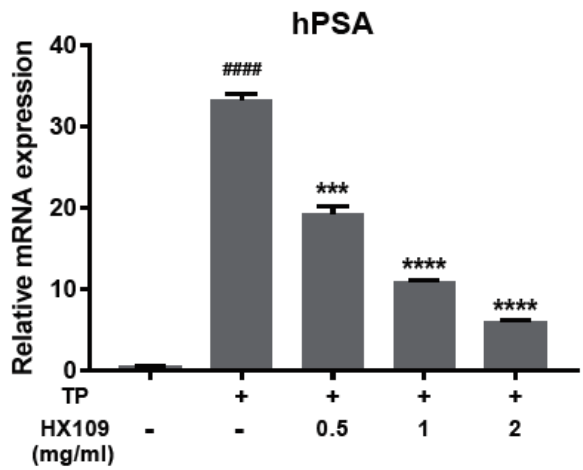


Figure 14. Effects of HX109 on hPSA mRNA expression. LNCaP cells were treated as described in Figure 11. The RNA levels were analyzed by quantitative RT-PCR after 24 h. Values were normalized to GAPDH for both protein and RNA analysis. $^{####}p<0.0001$ compared with control, *p<0.05, ***p<0.001, ****p<0.0001 (one-way ANOVA) compared with TP only. All data are represented as mean ± S.E.M of three independent experiments.

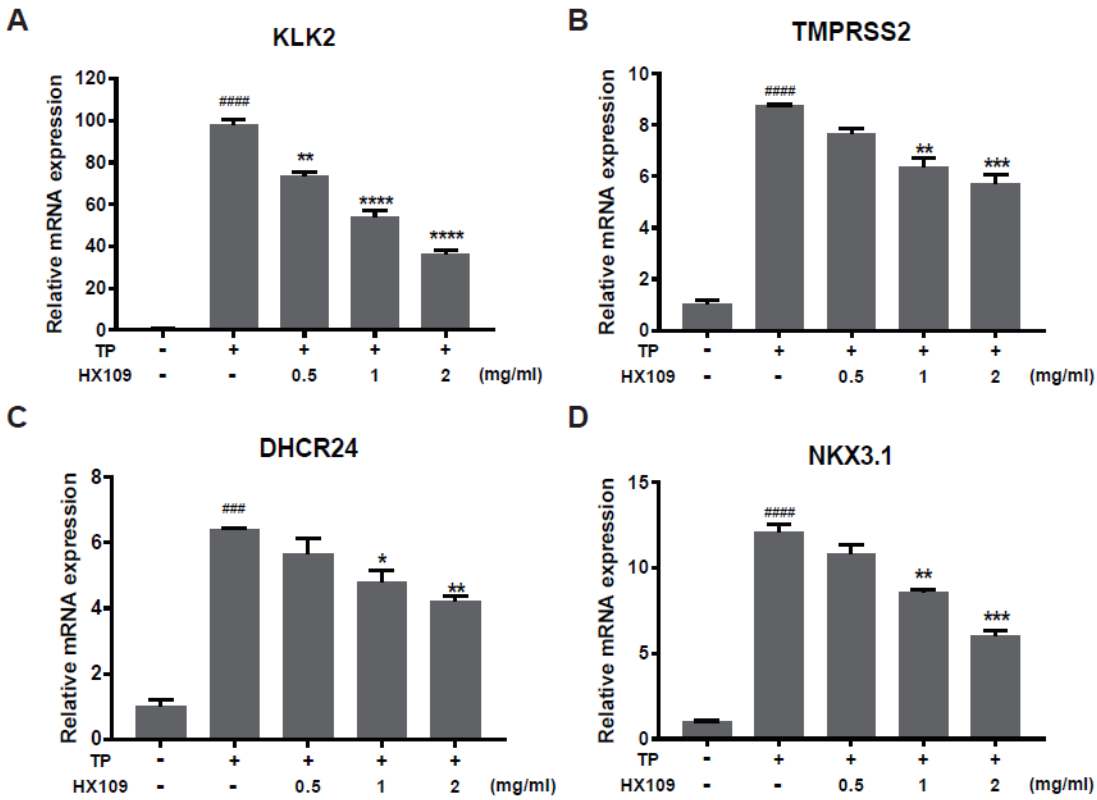


Figure 15. Effects of HX109 on androgen target genes. LNCaP cells were treated as described in Figure 11. The RNA levels of KLK2 (A), TMPRSS2 (B), DHCR24 (C) and NKX3.1(D) were analyzed by quantitative RT-PCR after 24 h. ####p<0.0001 compared with control, *p<0.05, ***p<0.001, ****p<0.0001 (one-way ANOVA) compared with TP only. All data are shown as mean ± S.E.M of three independent experiments.

expression.

2.5. HX109 Inhibits AR Transcriptional Activity Without Affecting AR Expression and Translocation

The induction of PSAs by androgen is mediated by the binding of AR to ARE present in the PSA promoter [60]. Therefore, AR-dependent transactivation was assessed by a reporter plasmid assay in which luciferase expression is dependent on the binding of AR to ARE element. LNCaP cells were transfected with luciferase reporter construct containing three copies of ARE, and then treated with TP in the presence of HX109. As shown in Figure 16, the level of luciferase activity was increased by TP, but was reduced by HX109 treatment in a dose-dependent manner.

It was also tested whether HX109 inhibited the expression of AR itself. No significant change in AR expression levels was observed in cells treated with HX109 for 24 h (Figure 17A). Lastly, the effects of HX109 on AR nuclear translocation were studied by isolating cytoplasmic and nuclear proteins. In the absence of TP, AR was only presented in the cytoplasmic compartment, but translocated to the nucleus upon TP treatment. There was little difference in the nuclear levels of AR between the TP-only and HX109 groups (Figure 17B). Taken together, these results suggest that HX109 might inhibit the transcriptional activity of AR without affecting AR expression or translocation.

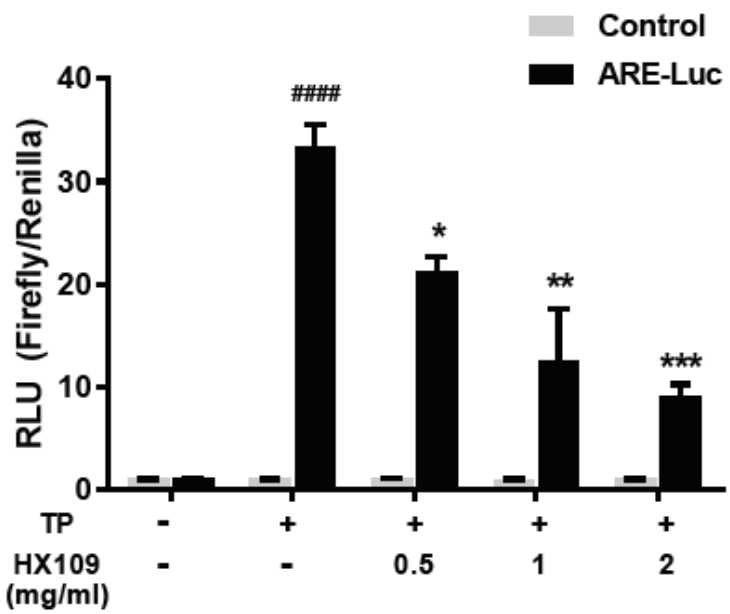


Figure 16. Effects of HX109 on ARE luciferase activity. LNCaP cells were transfected with control or luciferase reporter plasmid containing ARE sequence for 24 h. Transfected cells were treated with or without 100 nM TP in the presence of various concentrations of HX109 for 18h. Luciferase activity was measured as described in Material and Method. $^{\#\#\#\#}p<0.0001$ (one-way ANOVA) compared with control, $*p<0.05$, $***p<0.001$, $****p<0.0001$ (one-way ANOVA) compared with TP only. All values are shown as mean ± S.E.M of three independent experiments.

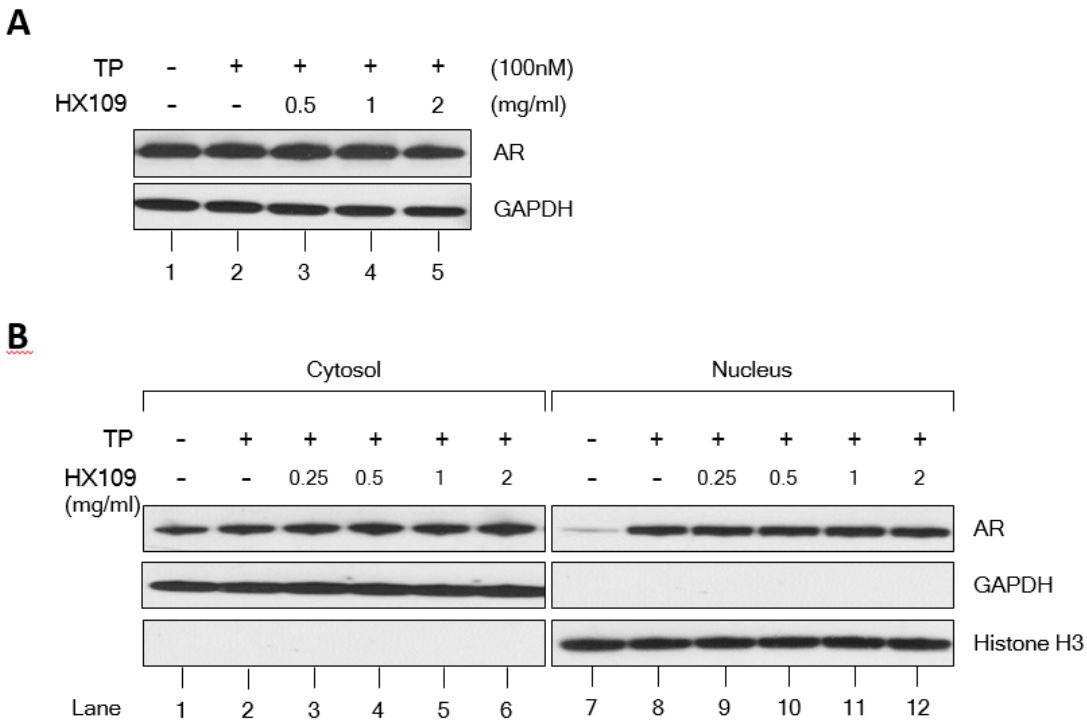


Figure 17. Effects of HX109 on AR expression and translocation. (A) The protein levels of AR were analyzed by Western blot after 24 h treatment with HX109. (B) Effects of HX109 on AR translocation. LNCaP cells were treated with 100 nM TP in the presence of various concentrations of HX109 for 3 h. After treatment, nuclear and cytoplasmic extracts were collected and subjected to Western blot. For Western blot, three independent experiments were performed, and one representative result is presented here.

2.6. HX109 Regulates AR Transactivation Through Upregulation of CaMKKββ and ATF3

It was previously reported that CaMKKβ and ATF3 repress the transactivation of AR [52, 53], and that the expression of the ATF3 gene is downregulated in human BPH tissue [61]. We first assessed the effect of HX109 on ATF3 expression. In LNCaP cells, TP had no effect on ATF3 expression, but treatment with HX109 increased the RNA (Figure 18A) and protein (Figure 18B) levels of ATF3 in a dose-dependent manner. To study the role of ATF3 in the HX109-mediated suppression of PSAs, ATF3 expression was inhibited using siRNA specific to ATF3. The knockdown efficiency of ATF3 was confirmed by western blot analysis (Figure 19A). Consistent with previous reports showing the repression of AR transactivation by ATF3, the inhibition of ATF3 expression increased TP-induced PSA mRNA expression. However, the inhibition of ATF3 expression interrupted PSA suppression by HX109 (Figure 19B).

Next, the effects ofHX109 on CaMKKβ activity were evaluated by western blot, as CaMKKβ is known to inhibit AR-mediated gene expression and AR transcriptional activity [53]. TP had no effects on CaMKKβ phosphorylation in LNCaP cells, but treatment with HX109 increased levels of phosphorylated CaMKKβ in a dose-dependent manner (Figure 20). When cells were pre-treated with the CaMKKβ inhibitor STO-609, HX109 did not affect PSA expression,

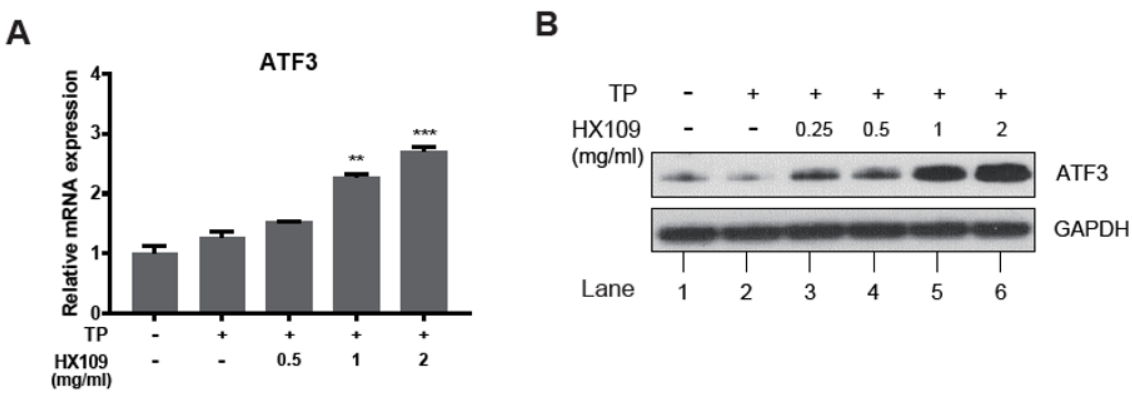


Figure 18. Effects of HX109 on ATF3 expression. LNCaP cells were treated as described in Fig 11, and total RNAs and proteins were prepared and analyzed for ATF3 by quantitative RT-PCR and Western blot, respectively. Effects of HX109 on ATF3 RNA (A) and protein (B) levels were measured after a 6 h or 8 h treatment with HX109, respectively. **p<0.01, ***p<0.001 (one-way ANOVA) compared with TP only.

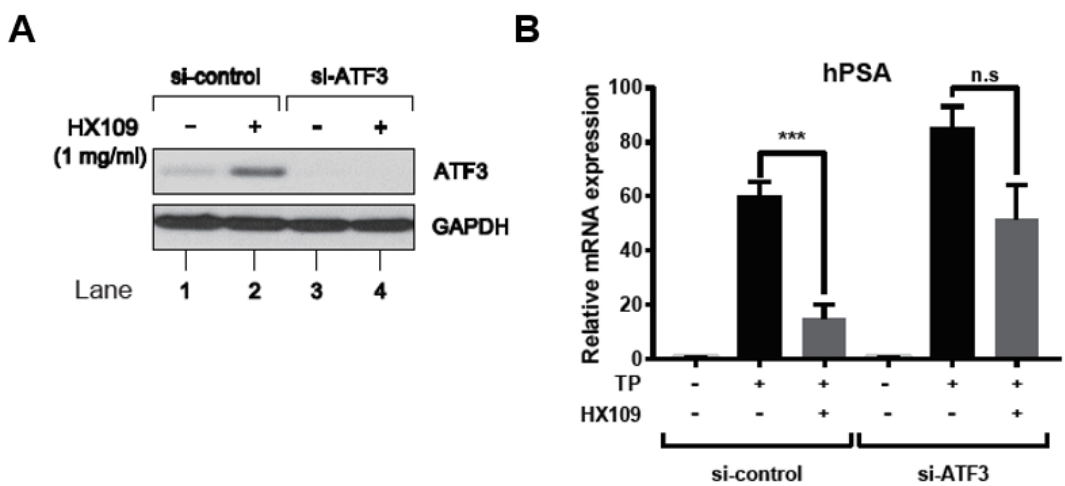


Figure 19. Effects of silencing ATF3 on PSA suppression by HX109. LNCaP cells were transfected with ATF3 siRNA or control siRNA for 24 h and then treated with 100 nM TP in the presence of HX109 1 mg/ml. (A) The protein levels of ATF3 were determined by Western blot after 8 h treatment. (B) The expression of hPSA RNA levels were measured by quantitative RT-PCR after 24 h treatment. Values of qRT-PCR were normalized to GAPDH. ***$p<0.001$ (one-way ANOVA) compared with TP only. n.s, not significant. For Western blot, three independent experiments were performed, and one representative result is shown here. All values are shown as mean ± S.E.M of three independent experiments.

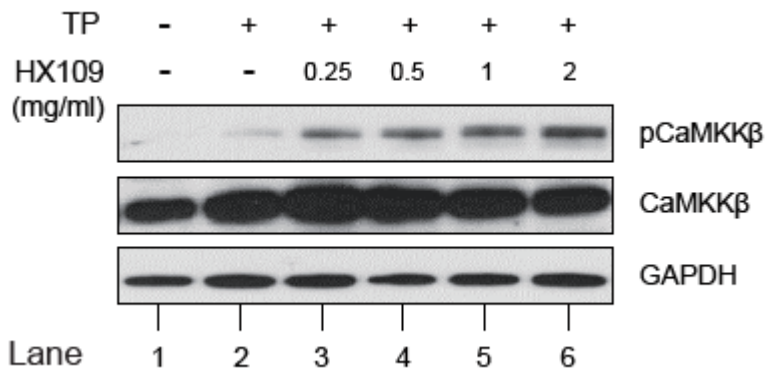


Figure 20. Effects of HX109 on phosphorylation of CaMKKβ. Total proteins were prepared after 30 min of TP and HX109 treatment and the phosphorylation of CaMKKβ were measured by Western blot. For Western blot, three independent experiments were performed, and one representative result is shown here.

showing the important role of CaMKKβ in the HX109-mediated inhibition of PSAs (Figure 21). Taken together, our data suggested that the inhibition of androgen-mediated gene expression by HX109 might be dependent on ATF3 and CaMKKβ.

2.7. Calcium Increase by HX109 Plays a Crucial Role in the Regulation of AR Transactivation

The binding of Ca2+/calmodulin to CaMKKβ increases its enzymatic activity. To test whether HX109 regulates intracellular calcium levels, calcium assays were performed using cell lysates. When cells were treated with HX109, intracellular calcium levels increased at 1 min and decreased at 5 min (Figure 22A). The role of calcium in HX109's effect on PSAs was confirmed in the Ca2+-free conditions. When cells were pretreated with the extracellular/intracellular calcium chelator BAPTA-AM, the HX109-mediated downregulation of PSA expression was inhibited (Figure 22B). However, pretreatment with EGTA, an extracellular specific calcium chelator, did not affect the effect of HX109 on PSA expression (Figure 23). These results indicated that HX109 might increase intracellular calcium to inhibit AR-mediated gene expression

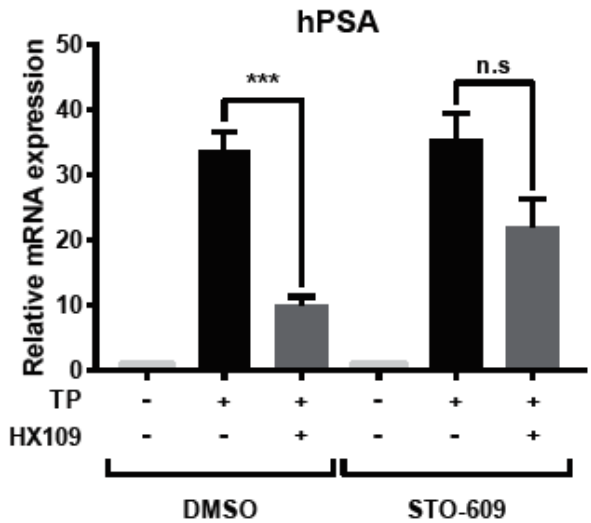


Figure 21. Effects of CaMKKβ inhibition on PSA suppression by HX109.

LNCaP cells were incubated with 30 μM STO-609 for 30 min and treated with 100nM TP in the presence of HX109 1 mg/ml for 24 h. The RNA levels of hPSA were measured by quantitative RT-PCR. Values of qRT-PCR were normalized to GAPDH. ***$p<0.001$ (one-way ANOVA) compared with TP only. n.s, not significant. All values are shown as mean ± S.E.M of three independent experiments.

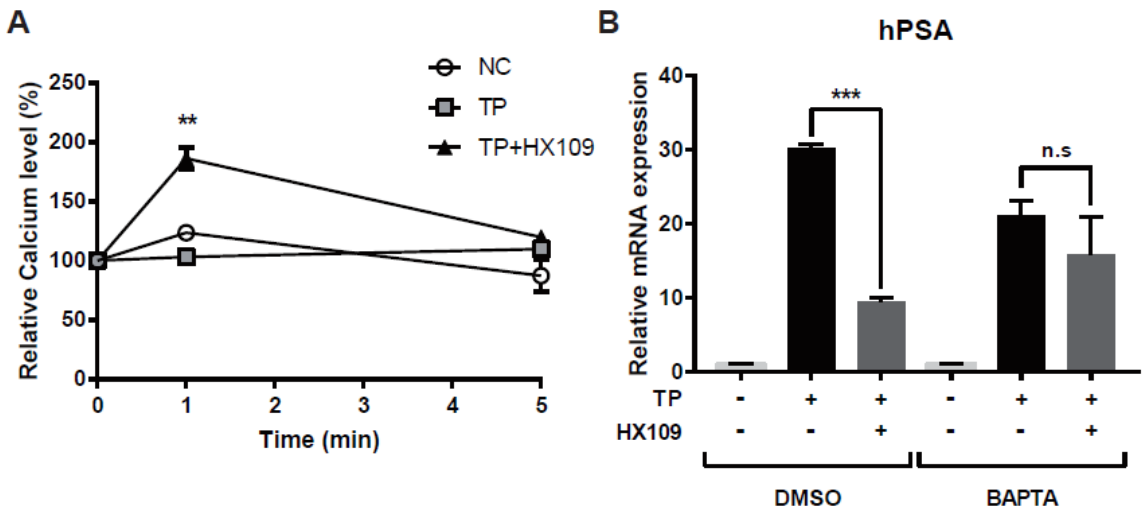


Figure 22. Role of Calcium increase by HX109 in the regulation of AR transcriptional. (A) Effects of HX109 on intracellular calcium levels. LNCaP cells were treated with 100 nM TP in the presence of HX109 1 mg/ml. Calcium levels were measured as described in Material and Method. **p<0.01 (one-way ANOVA) compared with TP only. (B) Effects of HX109 on calcium chelating condition. LNCaP cells were incubated with 20μM BAPTA-AM for 30 min and treated with 100 nM TP in the presence of HX109 1 mg/ml for 24 h. The RNA levels of hPSA were measured by quantitative RT-PCR. Values were normalized to GAPDH. ***p<0.001 (one-way ANOVA) compared with TP only. n.s, not significant. All Data are shown as mean ± S.E.M of three independent experiments.

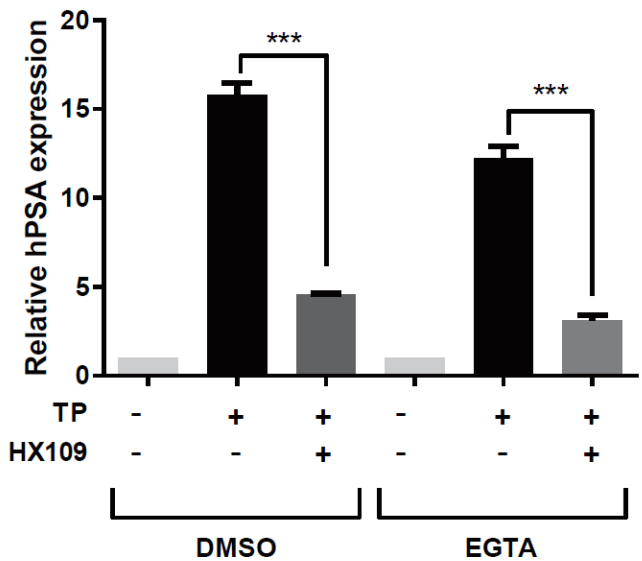


Figure 23. Effects of HX109 on extracellular calcium chelating condition.

LNCaP cells were incubated with 5 mM EGTA for 30 min and treated with 100 nM TP in the presence of HX109 1 mg/ml for 24 h. The RNA levels of hPSA were measured by quantitative RT-PCR. Values were normalized to GAPDH. ***$p<0.001$ (one-way ANOVA) compared with TP only. All Data are shown as mean ± S.E.M of three independent experiments.

3. Discussion

In this study, we showed that the botanical formulation HX109 control androgen receptor signaling. In LNCaP cells, HX109 inhibited androgen-induced proliferation and repressed androgen receptor-mediated gene expression. It appeared that these effects were mediated through an increase in the levels of ATF3 expression, phosphorylation of CaMKKβ, and also an increase in calcium levels, as the effects of HX109 were attenuated by treatment with ATF3-specific siRNA, CaMKKβ inhibitor, or calcium chelator.

Androgen binds to AR, translocates to the nucleus, and binds to ARE present in the promoters of various genes, eventually leading to cell proliferation. Various molecules known to inhibit androgen signaling exert their effects by blocking AR-androgen interaction, AR degradation, or AR translocation. However, HX109 did not seem to use conventional pathways to exert its effect. Instead, HX109 appeared to repress AR transactivation by upregulating the AR-interacting factors ATF3 and CaMKKβ. This indirect regulation of AR signaling by HX109 might have advantages, as it might produce lesser side effects than finasteride, which directly modulates AR signaling by inhibiting of DHT [62].

It has been reported that intracellular calcium levels are regulated by the influx of external calcium, by calcium channel openings, and by the release of calcium stored in endoplasmic reticulum (ER) [63]. In this study, pretreatment with EGTA, an extracellular calcium chelator, exerted no effect, while BAPTA-AM

suppressed HX109-mediated PSA reduction. Therefore, the HX109-mediated increase in intracellular calcium appears to be the result of calcium release from ER, rather than influx from the outside. It has been reported that Taraxacum officinale can raise calcium levels through the regulation of ER [64]. Further studies are needed to clarify the exact mechanism underlying the HX109-mediated regulation of calcium levels in the cells.

Increases in intracellular calcium levels resulting from HX109 action may activate CaMKKβ by increasing its phosphorylation, as evidenced by our data. Since CaMKKβ is well known to repress AR-mediated gene expression [53], the activation of CaMKKβ may play a crucial role in the HX109-mediated suppression of androgen signaling. In addition, CaMKKβ regulates the phosphorylation of AMP-activated protein kinase (AMPK), which has been shown to inhibit prostate cell growth and AR activity [65, 66]. Therefore, it is possible that HX109 may control AMPK through the activation of CaMKKβ, thereby producing the therapeutic effects observed in this study.

ATF3 is expected to target BPH pathogenesis by mitigating oxidative stress and inflammation, or inhibiting androgen signaling [67]. Furthermore, the levels of ATF3 expression in the prostates of BPH patients have been shown to be lower than those of healthy control groups [61]. We showed that HX109 upregulated ATF3 expression, suppressing AR-mediated gene expression and eventually prostate enlargement. It would be interesting to investigate how HX109 would

upregulate ATF3 expression at molecular levels.

Taken together, our data indicate that HX109 controlled AR-mediated gene expression and proliferation through the upregulation of ATF3, CaMKKβ, and intracellular calcium levels. The safety of the plants used for the preparation of HX109 has been established by a long history of human use. Our data suggest that HX109 may have the potential to be a safe and efficacious therapeutic agent for BPH.

Chapter IV

Anti-inflammatory effects of HX109 in macrophage-prostate crosstalk

1. Background

Despite the worldwide prevalence of BPH, the pathogenesis of the disorder is unclear, although several factors, such as aging, hormonal imbalance, chronic inflammation, and oxidative stress have been postulated [9]. Many recent studies show that prostatic inflammation is an important causative factor in BPH pathogenesis [27, 30, 31, 68]. In BPH patients, infiltrated lymphocytes and macrophages were commonly found in prostate tissue [32]; and the number of inflammatory infiltrates, mostly macrophages and T cells, is higher in aged mouse prostates [69].

Studies using co-cultures between macrophages and prostate cells have elucidated how these two cell types communicate with each other. Co-culture with macrophage increases cell proliferation of the prostate epithelial cells and stromal cells [70, 71]. In addition, macrophages play an important role in BPH development and progression by promoting the migration of prostate epithelial cells and EMT, a highly conserved cellular process[72, 73]. In the crosstalk process between the two cells, a variety of cytokines and chemokines are reported to be involved [74-76]. Macrophage activation in prostate tissue secretes a large number of different inflammatory cytokines to induce the inflammatory response as well as crosstalk with prostate cells. Since these proinflammatory cytokines enhance the proliferation of prostate epithelial cells and stromal cells [77-79], targeting the activated macrophage of prostate tissue may be a strategy for developing

therapeutic agents for BPH. AR has been known to play an important role in macrophage-prostate epithelial cell communication [80]. As such, this study, hypothesizes that HX109 might regulate crosstalk between macrophages and prostate epithelial cells. In addition, since *Taraxacum officinale* has been reported to contain anti-inflammatory activities [81-83], it was hypothesized that HX109 might also inhibit macrophage activation.

Here, we demonstrate that HX109 could inhibit macrophage-induced proliferation, migration, and EMT of prostate cell and macrophage activation. These results suggest that HX109 might be used to inhibit infiltrated macrophage-mediated prostate hypertrophy.

2. Results

2.1. HX109 inhibits macrophage induced prostate epithelial cell proliferation

To investigate the effects of HX109 on macrophage induced prostate epithelial cell proliferation, co-culture of macrophages and prostate epithelial cell lines were prepared and treated with 2 mg/ml of HX109. The presence of THP-1 increased proliferation of prostate epithelial cell line RWPE-1 cells by 34 % compared to the control group, which was inhibited when treated with 2 mg/ml HX109 (Figure 24A). To be certain that these inhibitory effects were not due to cytotoxicity, cells were treated with various concentrations of HX109.

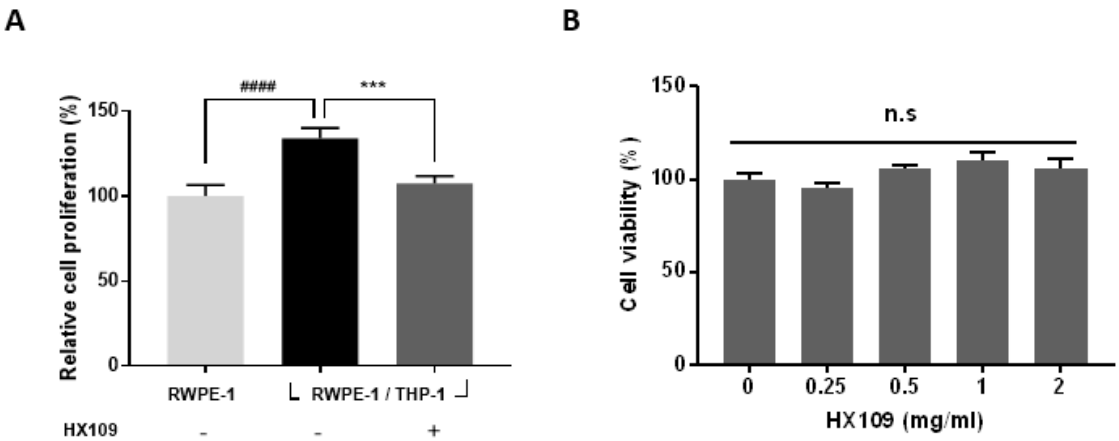


Figure 24. Effects of HX109 on macrophage-induced prostate epithelial cell proliferation. (A) Effects on cell proliferation. RWPE-1 cells were plated in culture media, and insert wells including THP-1 macrophages or control media were placed into each cell-seeded well, and incubated with or without 2mg/ml HX109 for 48 h. Cell proliferation was measured by WST-1 assay. (B) Effects on cell viability. RWPE-1 cells were cultured in the presence of various concentration of HX109 for 48 h. Cell viability was measured by WST-1 assay. $^{\#\#\#\#}p<0.0001$ (one-way ANOVA) compared with control, ***$p<0.001$ (one-way ANOVA) compared with RWPE-1/THP-1 co-culture. N.s, not significant. Values are normalized to control. All Data are shown as mean ± S.E.M of three independent experiments.

HX109 did not show cytotoxic effects in any of the concentrations. (Figure 24B). These data indicate that HX109 inhibited the macrophage-mediated proliferation of prostate cells.

2.2. HX109 suppresses prostate epithelial cell migration and epithelial-mesenchymal transition (EMT) in macrophage-prostate epithelial cell co-culture

It has been reported that prostate cell migration and EMT markers are increased in the co-culture of macrophages with prostate cells, resulting in the promotion of the development of prostatic hyperplasia [80]. To investigate the effect of HX109 on the macrophage-induced migration of prostate cells, THP-1 cells and RWPE-1 cells were co-cultured and a migration assay was performed. The migration ability of RWPE-1 cells, when co-cultured with THP-1, was increased by 89% compared to the control group, which was highly suppressed by treatment with 2 mg/ml of HX109 (Figure 25).

Co-culture of RWPE-1 cells with THP-1 cells has been known to induce the expression of various EMT-related genes in these epithelial cells [84]. Total RNA was prepared for 48 hours of co-culture followed by quantitative RT-PCR. The RNA levels of N-cadherin and snail were increased after co-culture, but were significantly reduced when treated with 2 mg/ml of HX109 (Figure 26). These results suggest that HX109 could inhibit macrophage-induced prostate epithelial cell migration and EMT gene expression.

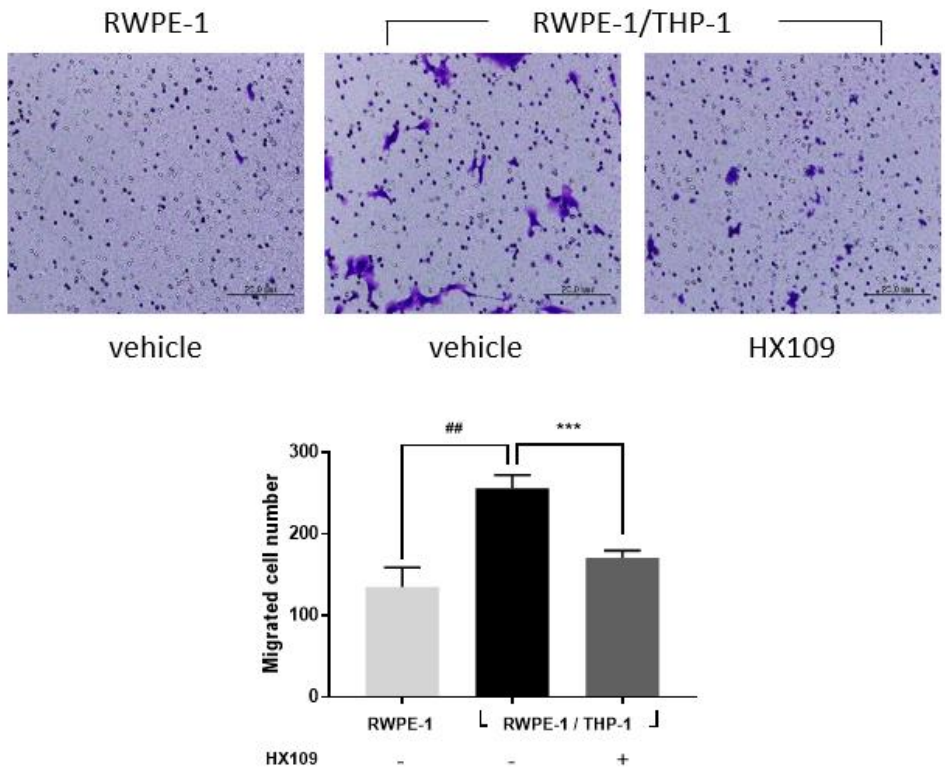


Figure 25. Effects of HX109 on epithelial cell migration in macrophage-prostate epithelial co-culture. RWPE-1 cells were seeded in the upper chamber of 8 um transwell plates and THP-1 macrophage or control medium was added to the lower chamber. Cells were incubated with or without 2mg/ml HX109 for 24 h. The cells migrated through pores were stained with 0.2% crystal violet and counted in 6 random fields. In the graph, the number of migrated cells was expressed as the average number of cells per field. $^{\#\#}p<0.01$ (one-way ANOVA) compared with control, ***$p<0.001$ (one-way ANOVA) compared with RWPE-1/THP-1 co-culture. All Data are shown as mean ± S.E.M of three independent experiments.

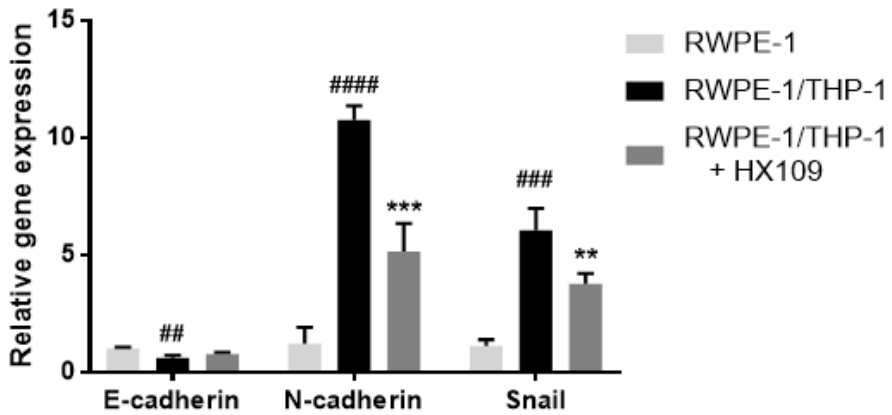


Figure 26. Effect of HX109 on the RNA level of EMT-related genes. The RNA levels of RWPE-1 cells 48 h after co-culture of with THP-1 macrophages in the presence of 2mg/ml HX109 were analyzed by qRT-PCR. Values of qRT-PCR were normalized to GAPDH. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ (one-way ANOVA) compared with control, $^{**}p<0.01$, $^{***}p<0.001$ (one-way ANOVA) compared with RWPE-1/THP-1 co-culture. All Data are shown as mean ± S.E.M of three independent experiments.

2.3. HX109 regulates macrophage-prostate epithelial cell crosstalk by inhibition of CCL4-STAT3 pathway

It has been shown that constitutively active forms of STAT3 promote EMT and the migration of prostate epithelial cells [85], so we examined the effect of HX109 on the STAT3 signaling pathway. RWPE-1 cells were co-cultured with THP-1 cells for 24 hours, and total proteins were prepared followed by Western hybridization analysis. As shown in Fig 27, co-culture of the two cell types increased the level of phosphorylated STAT3 and its downstream gene, COX-2 (Figure 27). When treated with HX109 2mg/ml, however, the levels of pSTAT3 and COX2 which had been increased by co-culture was reduced.

It was reported that chemokines such as CCL2, CCL3 and CCL4 are involved in the regulation of STAT3 signaling in co-cultures between macrophages and prostate epithelial cells [84, 86, 87] . To determine which mediators are involved in the HX109-mediated regulation of STAT3 signaling, we measured the RNA levels of these chemokines. Among the three chemokines whose levels were increased in the co-culture, only CCL4's expression was inhibited by HX109 (Figure 28). These results suggest that HX109 might inhibit STAT3 signaling through the regulation of CCL4 levels during macrophage-prostate epithelial cell crosstalk.

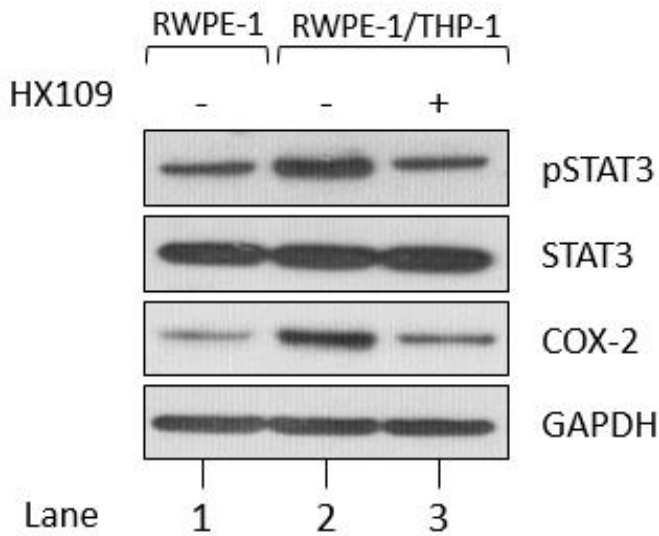


Figure 27. Effects of HX109 on STAT3 pathway. RWPE-1 cells were plated in culture media and insert wells including THP-1 macrophage or control media were put into each cell-seeded well, and incubated with or without 2mg/ml HX109. Total proteins were prepared after 24 h co-culture and analyzed for the protein levels of pSTAT3, STAT3, COX-2 and GAPDH proteins were used as loading control. For western blot, three independent experiments were performed, and one representative result is shown here

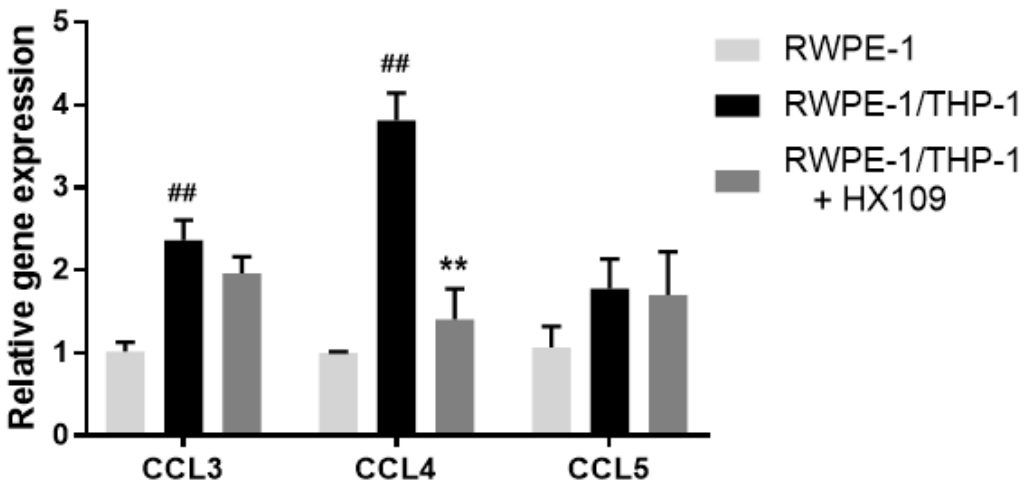


Figure 28. Effects of HX109 on chemokines mRNA expression levels. RWPE-1 cells were co-cultured with THP-1 as described in Figure 27. The RNA levels of RWPE-1 cells after 48 h were analyzed by qRT-PCR. Values of qRT-PCR were normalized to GAPDH. $^{\#\#}p<0.01$(one-way ANOVA) compared with control, **$p<0.01$ (one-way ANOVA) compared with RWPE-1/THP-1 co-culture. Values are normalized to control. All Data are shown as mean ± S.E.M of three independent experiments.

2.4. HX109 suppresses macrophage activation by inhibiting TAK1-IKK-Ikb-NF-kB pathway.

Next, we investigated the effects of HX109 on macrophage activation at the molecular level, using two macrophage cell lines, THP-1 and Raw264.7. In LPS-stimulated THP-1 cells, both RNA and protein levels of IL-6 and TNFα increased radically (Figure 29), but treatment with HX109 lowered the levels of both inflammatory cytokines in a concentration-dependent manner. Similar results were also obtained when Raw264.7 cells were used (Figure 30).

Regulation of NF-κB phosphorylation via Toll-Like-Receptor 4 (TLR4) is a key feature in the LPS-induced inflammatory response in macrophages [88]. To test whether HX109 controls NF-κB, THP-1 cells were treated with LPS and HX109 for 30 min, and the levels of NF-κB signaling proteins were measured by Western blot hybridization. LPS stimulation increased the levels of phosphorylated TGF-β activated kinase 1 (TAK1), IκB kinase (IKK) and p65, but co-treatment with HX109 lowered of these proteins in a concentration dependent manner (Figure 31). On the other hand, IκBα levels, which had dropped after LPS treatment, were recovered after HX109 treatment in a concentration-dependent manner (Figure. 31) These results showed that HX109 could suppress macrophage activation and the induction of inflammatory cytokines by inhibiting the TAK1-IKK-IκBα-NF-κB pathway.

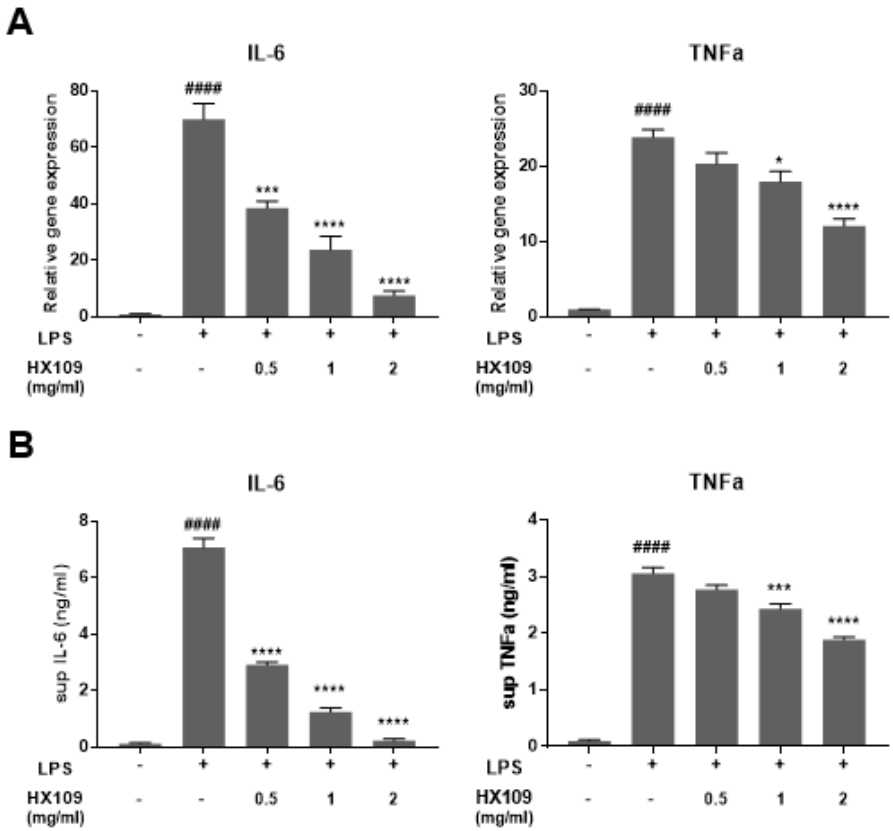


Figure 29. Effects of HX109 on activated macrophage in THP-1 cells. THP-1 macrophages were treated with or without 100ng/ml LPS and cultured in presence of various concentration of HX109. (A) The RNA levels of THP-1 cells after 3 h of LPS and HX109 treatment were analyzed by qRT-PCR. Values of qRT-PCR were normalized to GAPDH. (B) The protein level of inflammatory cytokines was analyzed by ELISA after 24 h.$^{\#\#\#\#}p<0.0001$ (one-way ANOVA) compared with control, $*p<0.05$, $***p<0.001$, $****p<0.0001$ (one-way ANOVA) compared with LPS only. All Data are shown as mean ± S.E.M of three independent experiments.

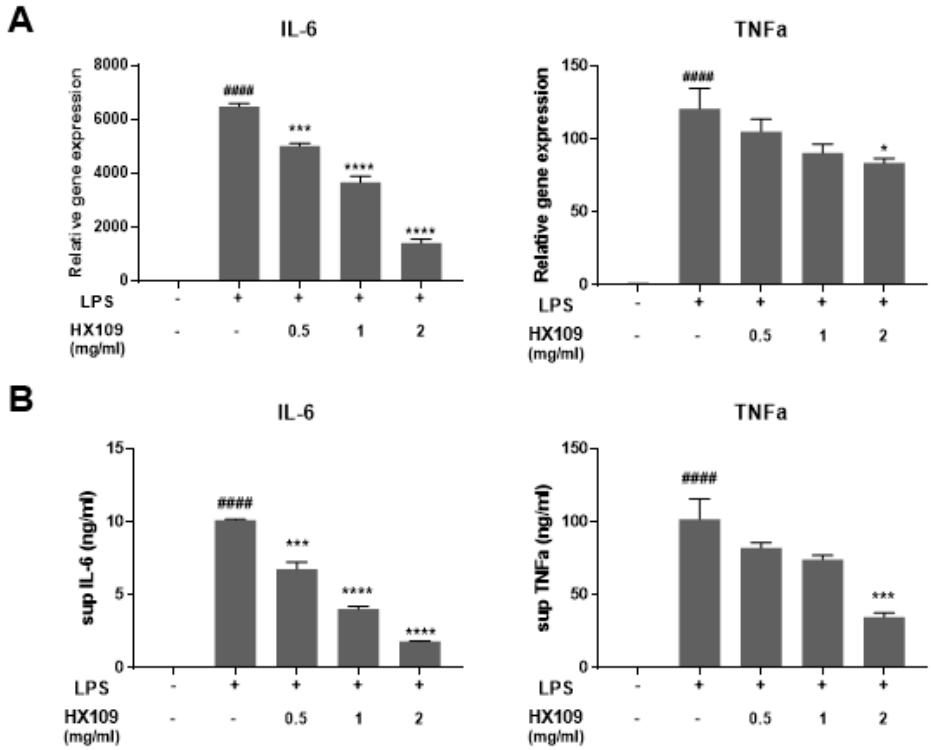


Figure 30. Effects of HX109 on activated mouse macrophage Raw264.7 cells. Raw264.7 cells were treated with or without 100ng/ml LPS and cultured in presence of various concentration of HX109. (A) The RNA levels of Raw264.7 cells after 3 h of LPS and HX109 treatment were analyzed by qRT-PCR. Values of qRT-PCR were normalized to GAPDH. (B) The protein level of inflammatory cytokines was analyzed by ELISA after 24 h. $^{####}p<0.0001$ (one-way ANOVA) compared with control, $*p<0.05$, $***p<0.001$, $****p<0.0001$ (one-way ANOVA) compared with LPS only. All Data are shown as mean ± S.E.M of three independent experiments.

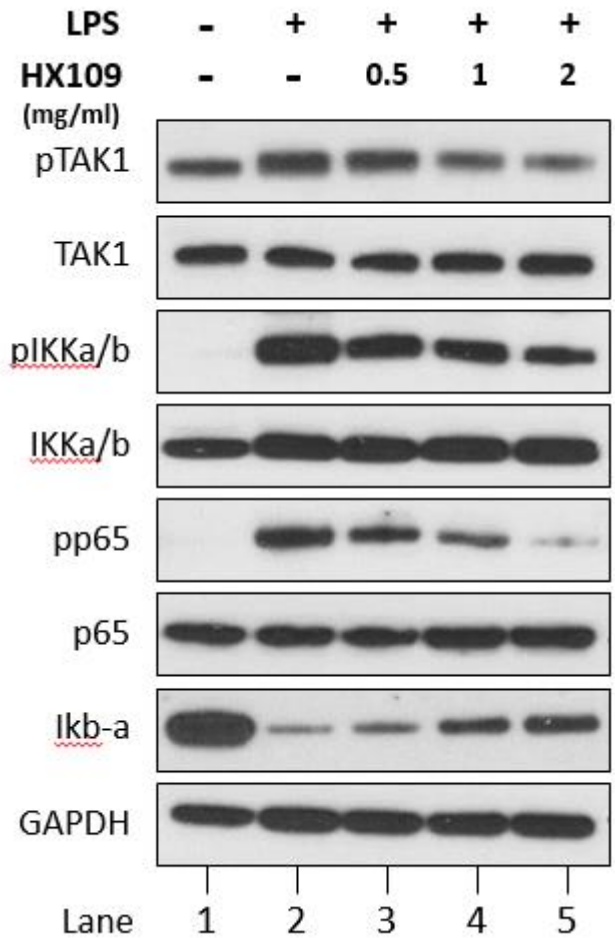


Figure 31. Effects of HX109 on p65 NF-κB signaling pathway. THP-1 macrophages were treated with or without 100ng/ml LPS and cultured in presence of various concentration of HX109. Total proteins of THP-1 macrophage were prepared after 30 min of LPS and HX109 treatment and analyzed by western blot. For western blot, three independent experiments were performed, and one representative result is shown here.

3. Discussion

In this study, we show that the botanical extract HX109 regulates crosstalk between macrophages and prostate cells and macrophage-mediated inflammation. In co-culture experiments, HX109 controlled macrophage-induced proliferation and the migration of prostate epithelial cells, and also inhibited EMT. Data from protein and RNA analyses indicate that these effects were mediated through the inhibition of CCL4-STAT3 signaling in prostate epithelial cells. In addition, HX109 suppressed the expression of inflammatory cytokines in activated macrophages by controlling NF-κB signaling.

It is well established that inflammation plays important roles during the pathogenesis of prostatic hyperplasia [21, 24, 25]. For example, it promotes the infiltration of immune cells like macrophage, and induces the expression of a variety of cytokines and chemokines that influence the proliferation of prostate cells [28, 29, 75]. Our data reveals that HX109 could exert multiple effects to regulate the pathogenies of BPH. Firstly, HX109 could inhibit the effects of macrophages on prostate epithelial cells at the cellular as well as gene expression levels. It appears that in epithelial cells, CCL4 and STAT3 signaling are the main target of HX109. Secondly, HX1019 seems to directly target macrophages to suppress their inflammatory effects as demonstrated by the effective reduction of the protein levels of phosphorylated TAK1, IKK, and p65 NF-κB and increase in that of IκBα. In summary, HX109 simultaneously targets two key cell types

involved in BPH pathogenesis, indicating that it might be an ideal starting point for developing safe and effective therapeutic agents.

In BPH pathogenesis, crosstalk between prostate and immune cells, particularly macrophages, has been reported to be important. In this process, AR is shown to play a key role by controlling the expression of several chemokines that mediate crosstalk and increase prostate hypertrophy [70]. Indeed, knock-out of AR resulted in decrease of macrophage infiltration [87]. Therefore, the regulation of the CCL4-STAT3 pathway by HX109 may have resulted from the inhibition of AR signaling by HX109. The understanding of how HX109 does this would be important in developing agents that control crosstalk between the two major cell types involved in BPH pathogenesis.

The *in vitro* co-culture systems used in this study mimic crosstalk between infiltrated macrophage and prostate epithelial cell. In this context, it is worth noting that the most commonly used prostatic hypertrophy models are induced by excessive androgen, which do not cause inflammatory responses or the infiltration of immune cells in the prostate [89]. To study crosstalk in vivo, it would be necessary to use a mouse model showing macrophage infiltration to the prostate, such as the recently developed prolactin-induced BPH model [90].

Chapter V

Concluding remarks

Benign prostatic hyperplasia (BPH) is a common disease in the elderly male population throughout the world. Since BPH symptoms seriously diminish the quality of life, there is an acute need for the development of safe, effective, and long-lasting therapeutic agents. In the research I conducted for this thesis, I developed HX109, a botanical formulation prepared from three plants (*Taraxacum officinale, Cuscuta australis, and Nelumbo nucifera*) to target BPH. These plants were selected on the basis of previous publications detailing their activities and functions, and information available from traditional phytomedical practices in Asia for targeting BPH symptoms.

In this study, I have shown that oral administration of HX109 could ameliorate TP-induced conditions in the BPH rat model. In the HX109-treated group, prostate weight and PSA levels were reduced, and prostatic acinar area was decreased in histological analysis. The BPH rat model used in this study was induced by prostate cell proliferation by TP, suggesting that HX109 inhibits prostate enlargement by inhibiting AR signaling. Results from *in vitro* experiments show that HX109 inhibited androgen-induced proliferation and repressed androgen receptor-mediated gene expression in LNCaP cells. Interestingly, unlike various molecules known to inhibit androgen signaling by blocking AR-androgen interaction, AR degradation, or AR translocation, HX109 inhibited androgen-dependent proliferation and expression of androgen target genes without using conventional pathways. Instead, HX109 appeared to repress AR transactivation by upregulating the AR-interacting factors ATF3 and CaMKKβ, a fact that was

demonstrated in experiments with si-RNA and inhibitors (Figure 32). Commonly used remedies like finasteride or anti-androgens directly inhibit AR signaling and therefore have various side effects. Thus, this indirect regulation of AR signaling by HX109 might be a great advantage when considering the compound as a replacement for existing therapeutics. Another interesting point is that HX109 increased intracellular calcium levels. In experiments using EGTA and BAPTA-AM, the HX109-mediated increase in intracellular calcium appears to be the result of calcium release from the ER, rather than an influx from the outside. Because calcium levels have a variety of cellular effects, further studies are needed to elucidate the effects of HX109 on calcium regulation and its underlying mechanism.

Next, a co-culture system involving macrophage-prostate epithelial cells was used to determine the effect of HX109 on inflammation in BPH development. HX109 could inhibit the effects of macrophages on prostate epithelial cell proliferation and migration, as well as EMT gene expression. It appeared that in macrophage-prostate epithelial cell crosstalk, CCL4 and STAT3 signaling in epithelial cells are major targets of HX109. Since AR is reported to play an important role by controlling the expression of several chemokines like CCL4 in this crosstalk, the regulation of the CCL4-STAT3 pathway by HX109 may have resulted from the inhibition of AR signaling by HX109. It was further investigated whether HX109 inhibits the inflammatory response in the activated macrophage. HX109 seems to directly target macrophages to suppress their inflammatory effects

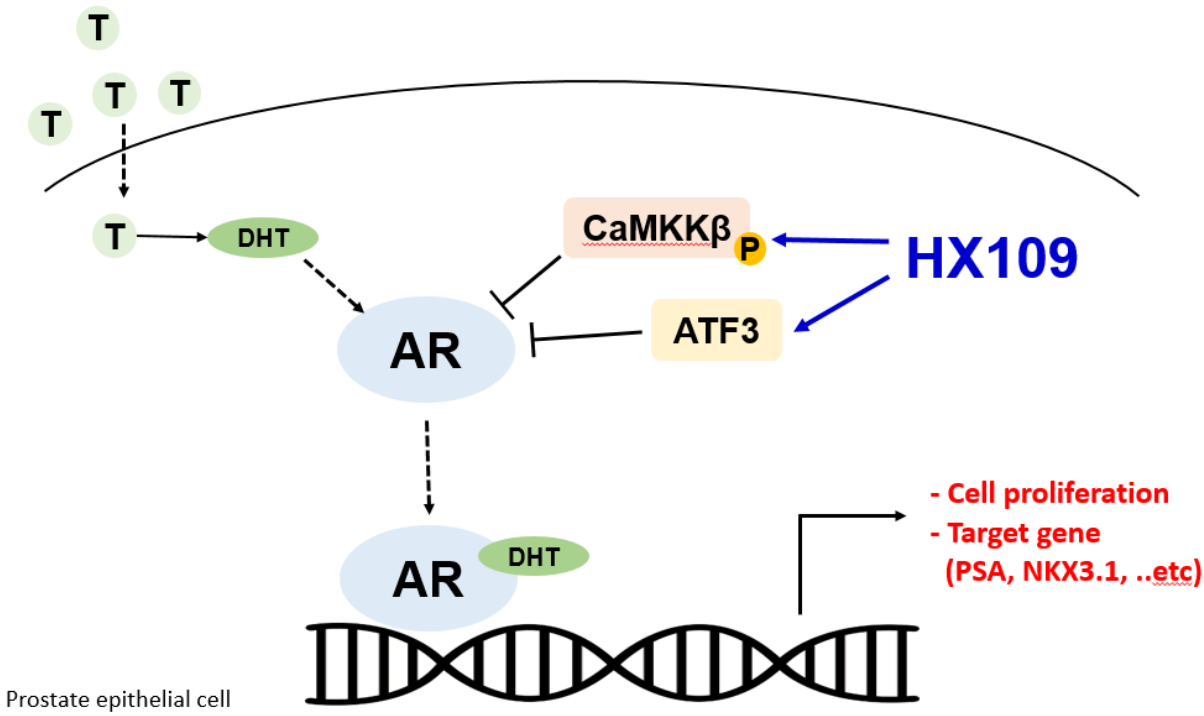


Figure 32. schematic diagram of mechanism underlying downregulation of AR signaling by HX109.

as demonstrated by the effective reduction of the protein levels of phosphorylated TAK1, IKK, and p65 NF-κB; and increase in that of IκBα (Figure 33). It remains to be elucidated whether the impact of HX109 on inflammation directly contributed to the formulation's therapeutic effects on BPH. The *in vivo* model used in this study is induced by androgen excess, which does not cause inflammatory responses or the infiltration of immune cells in the prostate. To investigate the effects of crosstalk regulation by HX109 on BPH development, further studies will be needed using a mouse model that shows macrophage infiltration to the prostate, such as the recently developed prolactin-induced BPH model.

I have not yet identified the compounds responsible for the observed effects of HX109 described in this study. Since HX109 consists of a wide variety of components, its effects might result from the complex interactions of multiple components rather than of one specific compound. For instance, flavonoids like quercetin and astragalin from *Cuscuta australis* have been shown to reduce oxidative stress in various cell types [91-93], and 7-hydroxydehydronuciferine and dauricine from *Nelumbo nucifera* have been reported to inhibit the proliferation of prostate cancer cells and urinary tract tumor cells [94, 95]. Therefore, the complex action of the various compounds contained in HX109 may result in an inhibition of prostate enlargement. For this reason, the greatest challenge of developing botanical therapeutics as medicines is their multifarious nature, which makes it difficult to pinpoint the biologically responsible compounds. There are two ways to overcome this obstacle. One is to identify all the compounds present in an extract

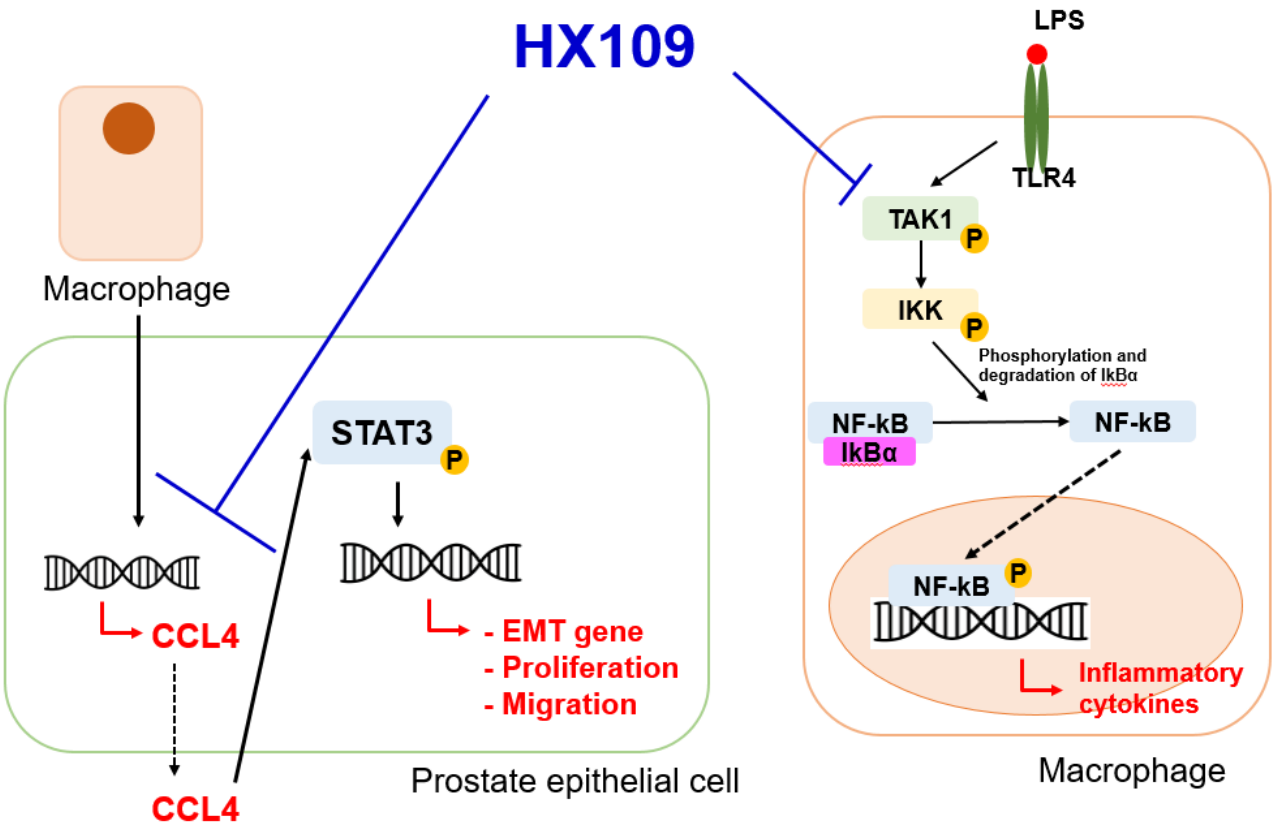


Figure 33. Schematic diagram of mechanism(s) underlying anti-inflammatory effects of HX109 in prostate.

and match the respective chemicals to particular biological activities. Although no one has shown convincing results with this approach, the recent developments in massive high-throughput analytical devices along with advances in informatics technology may make this option more viable than before. For example, the novel chemical knock-out strategy has been proposed to identify active compounds in herbal medicines. Similar to the idea of knocking out genes to study their function in cells, this strategy selectively removes each component in the herbal medicine using preparative HPLC [96, 97]. The other approach, which we take in this research, is to accept the complex nature of a "mixture" as it is, and use cell-based bioassays to ensure the consistency of the extract in the context of bioactivities. As described in ChapterⅢ, HX109 was prepared in a reproducible manner by developing cell-based bioassay using human PSA as a biomarker. Further studies identifying the active compounds, or at least the fraction with concentrated bioactivity, are warranted before moving on to the pharmaceutical development of this botanical extract.

In conclusion, the data shown in this thesis suggest that HX109 ameliorates TP-induced prostate enlargement and histological development. HX109 controls AR-mediated gene expression and proliferation through the upregulation of ATF3, CaMKKβ, and intracellular calcium levels. At the same time, I have shown that HX109 inhibits increased levels of proliferation, migration, and EMT gene expression through the downregulation of the CCL4-STAT3 pathway in

macrophage-prostate epithelial cell crosstalk. In addition, HX109 controls the production of inflammatory cytokines in macrophages by suppressing NF-κB signaling. Taken together, it appears that HX109 may be developed as a safe and effective agent for the treatment of BPH.

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국문초록

전립선 비대증(BPH)은 전세계적으로 노인 남성 인구에서 흔한 질병이다. 전립선 비대증의 원인은 아직 완전히 밝혀지지 않았지만, 질병의 발달에는 노화, 호르몬 변화, 대사 증후군, 산화 스트레스, 염증 등의 다양한 요인들이 관여한다. 현재 5-알파 환원효소 억제제 (5ARI)나 알파차단제와 같은 약들이 사용되고 있지만, 이들은 여러 부작용을 가지고 있기 때문에 안전하고, 효과적이며, 오래 지속되는 치료제의 개발이 필요하다.

전립선 비대증은 여러 요인에 의해 발병하기 때문에, 식물소재는 치료제 개발을 위한 흥미로운 소재가 될 수 있다. 여러 구성성분을 포함하고 있는 식물소재는 이 성분들이 다양한 세포표적과 상호작용하기 때문에 이점을 갖는다. 그러므로 본 연구에서는 전립선 비대증을 치료하기 위하여 3가지 식물소재 (포공영, 토사자, 연자육)로부터 제조된 식물제재인 HX109를 개발하였다. 이 3가지 식물의 혼합물로 에탄올 추출물을 제조하였고, 세포 기반 생물 검정 방법과 고성능 액체 크로마토그래피 (HPLC)를 이용한 몇몇 지표 성분 정량에 의해 그 품질을 관리하였다. 이 연구에서, HX109가 전립선 비대증에서 개선

효과를 나타낼 수 있다는 가설을 세웠다. 또한, 본 논문 연구는 HX109의 메커니즘을 밝히기 위해 HX109의 안드로겐 신호전달 억제 및 항 염증 효과에 중점을 두었다.

먼저, 테스토스테론 프로피오네이트(TP)로 유도한 전립선 비대증 랫드 모델에 대한 HX109의 효과를 알아보았다. HX109의 경구투여는 TP에 의해 유도 된 전립선 비대 및 조직학적 변화를 개선시켰다. 인간 전립선 상피세포주인 LNCaP 세포에서, HX109는 안드로겐 수용체에 의해 매개된 세포 증식을 억제하지만 표피 성장 인자 (EGF)에 의해 매개된 세포 증식은 억제하지 않았다. 또한, HX109는 AR의 핵 전위 또는 발현에 영향을 미치지 않으면서 전사 수준에서 AR 표적 유전자의 유도를 억제 하였다. AR 신호 전달에 대한 HX109의 이러한 효과는 활성화 전사 인자 3 (ATF3) 활성화 및 칼슘/칼 모듈린-의존성 단백질 키나아제 키나아제 β (CaMKKβ)의 인산화의 조절을 통해 매개되었으며, ATF3 특이적 siRNA 및 CaMKKβ 억제제를 이용한 실험의 데이터로 각각 입증되었다. 이러한 결과는 HX109가 AR 신호 전달의 억제를 통해 BPH의 증상을 개선 할 수 있음을 나타냈다.

다음으로, BPH 발병의 중요한 원인 인자로 간주되는 염증에 대한 HX109의 효과를 THP-1 대식세포 세포주를 사용하여 조사 하였다. THP-1 대식세포 및 RWPE-1 전립선 상피 세포주를 포함하는 공동

배양 시스템에서, HX109는 대식세포에 의해 유도된 세포 증식, 이동 및 상피-중간엽 이행 (EMT)를 억제 하였다. 이러한 억제는 CCL4의 발현 및 STAT3의 인산화를 억제함으로써 매개된다. 또한, HX109는 염증성 사이토 카인의 발현 및 p65 NF-κB의 인산화를 농도 의존적으로 억제하였다. 이러한 결과는 HX109가 대식세포 활성화 및 전립선 세포와의 상호작용을 조절하고, 이를 통하여 BPH를 억제 할 수 있음을 시사한다.

결론적으로, HX109는 TP-유도 전립선 비대증 랫드 모델에 대한 치료 효과를 보여 주었다. 이러한 효과는 ATF3 및 CaMKKβ의 상향 조절을 통해 AR 신호 전달을 억제하거나 대식세포의 활성화 및 전립선 세포와의 상호작용을 억제함으로써 나타날 수 있다. 선행 및 본 논문 연구의 결과들을 종합해 볼 때, HX109는 BPH의 치료를 위한 안전하고 효과적인 치료제로서 개발될 가능성을 제시하였다.

핵심어: 전립선비대증, 식물 의약품, HX109, 테스토스테론, 안드로겐 수용체, 대식세포, 염증

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