PHYTOTHERAPY RESEARCH
Phytother. Res. (2016)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.5611

Blockade of Androgen Markers Using a Novel Betasitosterol, Thioctic Acid and Carnitine-containing Compound in Prostate and Hair Follicle Cell-based Assays

Li Chen,[1] Jiaolong Wang,[1] Glen Mouser,[2] Yan Chun Li[1]* and Geno Marcovici[3]*

[1]Department of Medicine, Division of Biological Sciences, University of Chicago, Chicago, IL, USA
[2]R&D Department, Omega Tech Labs, Boise, ID, USA
[3]R&D Department, Advanced Restoration Technologies Inc., Houston, TX, USA

Androgenetic alopecia (AGA) affects approximately 70% of men and 40% of women in an age-dependent manner and is partially mediated by androgen hormones. Benign prostatic hyperplasia (BPH) similarly affects 50% of the male population, rising by 10% each decade. Finasteride inhibits 5-alpha reductase (5AR) and is used to treat both disorders, despite offering limited clinical benefits accompanied by significant adverse side effects. Building on our previous work demonstrating the efficacy of naturally derived 5AR inhibitors (such as stigmasterol and beta sitosterol), we hypothesize that targeting 5AR as well as inflammatory pathways may yield improved efficacy in AGA and BPH. Here we address these dual pathomechanisms by examining the potency of a novel composition using *in vitro* assays of representative cell lines for AGA (hair follicle dermal papilla cells) and BPH (LNCaP prostate cells), respectively. Exposure of cells to the novel test composition down-regulated mRNA expression profiles characteristic of both disease processes, which outperformed finasteride. Changes in mRNA expression were corroborated at the protein level as assessed by western blotting. These studies provide proof of concept that novel, naturally derived compositions simultaneously targeting 5AR and inflammatory mediators may represent a rational approach to treating AGA and BPH. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: botanicals; phytotherapy; androgenetic alopecia; 5-alpha reductase.

INTRODUCTION

The crucial role of androgens in the development of a number of diseases is well established. Among these, benign prostatic hyperplasia (BPH) is thought to affect 210 million men worldwide (Wroński, 2014). BPH is the most common prostate problem for men older than age 50 (Lee and Lee, 2014). In 2012, as many as 14 million men in the USA had lower urinary tract symptoms suggestive of BPH (Radomski, 2014). Although BPH rarely causes symptoms before age 40, the occurrence and symptoms increase with age. BPH affects about 50% of men between the ages of 51 and 60 and up to 90% of men older than 80 (Corona *et al.*, 2014).

Likewise, androgenetic alopecia (AGA) progresses with age, but occurs in women as well as men. AGA ultimately affects 70% of men and 40% of women at some point in their lifetimes. Men present with a distinctive pattern involving hairline recession and vertex balding (Norwood-Hamilton classification). Women exhibit diffuse hair thinning over the crown (increased part width) and retention of the frontal hairline (Ludwig classification) (Vujovic and Del Marmo, 2014). The treatment of BPH and AGA presents challenging obstacles. Several lines of evidence link androgen receptor pathways to BPH and AGA and the use of androgen reducing compounds, such as 5α-reductase (5AR) inhibiting drugs, constitutes a key medical therapy in the standard of care for both BPH and AGA. Widely prescribed pharmaceutically based per os 5AR inhibitors, such as finasteride, offer limited clinical benefit while engendering multiple negative risk factors in men and are not FDA-approved for use in women.

In 5-mg oral dosage, finasteride, a selective type-2 5AR inhibitor, is the first drug in this class approved for BPH by the US Food and Drug Administration, and it remains the most widely prescribed 5AR inhibiting drug against BPH (McElwee and Shapiro, 2012). In human trials, finasteride decreased the prostatic DHT level by 70–90% and reduced prostatic volume (Kumar and Wahane, 2008). A serendipitous finding was the anecdotal report of thickened scalp hair in some BPH patients simultaneously affected by pattern hair loss (Chen *et al.*, 1996). Finasteride is thought to enhance hair growth because of its 5AR blockade mechanism. In 1996, finasteride, in 1-mg oral dosage, was approved for the treatment of males suffering AGA. Subsequent, widely reported negative side effects of finasteride include loss of libido, feminizing birth defects, suicidal ideation, and gynecomastia (Traish *et al.*, 2014).

* Correspondence to: Geno Marcovici, Ph.D., Advanced Restoration Technologies, Inc., 3730 Kirby, Suite 1200, Houston, TX 77098, USA; Yan Chun Li, Ph.D., Department of Medicine, The University of Chicago, 900 E. 57th Street, KCBD 9110, Chicago, IL 60637, USA.
E-mail: dr.marcovici@hairgenesis.com (Geno Marcovici); cyan@medicine.bsd.uchicago.edu (Yan Chun Li)

Received 04 November 2015
Revised 21 February 2016
Accepted 24 February 2016

Previously, we demonstrated the efficacy of naturally derived 5AR inhibitors incorporating stigmasterol and beta sitosterol in a series of placebo controlled and open-label clinical trials of men with AGA. We then extended this formulation and further incorporated carnitine and thiocitic acid, which in addition to blocking 5-alpha reductase, also exhibited antiinflammatory characteristics in a well-established *in vitro* assay representing hair follicle keratinocyte gene expression (Chittur *et al.*, 2011). We postulate that the blockade of inflammation in the skin represents a new and potentially viable therapeutic avenue for the treatment of AGA. This hypothesis is supported with several lines of evidence, including cutaneous immunopathology which directly implicates inflammation as a factor in AGA (Young *et al.*, 1991) (Yoon and Baek, 2005).

In this study, we tested a naturally derived composition with 5AR inhibitory and antiinflammatory characteristics against the benchmark drug finasteride in a series of *in vitro* assays that recapitulate the cellular microenvironment of AGA.

MATERIALS AND METHODS

Cell culture and treatment. LNCaP cells were obtained from ATCC (Manassas, VA) and cultured in DMEM supplemented with 10% FBS at 37 °C and 5% CO_2. Human follicle dermal papilla cells (HFDPCs) were purchased from PromoCell GmbH (Heidelberg, Germany) and cultured at 37 °C and 5% CO_2 in the HFDPC growth medium supplied by PromoCell. For dose response experiments, cells at confluence were treated with finasteride (Sigma-Aldrich) at a dose range from 0 to 10 μM, or the test compounds at 0–250 μg/mL for 24 h. Test compound was composed of beta sitosterol powder (97% purity), stigmasterol (95% purity), acetyl-L-carnitine powder (99% purity), and thioctic acid powder (99% purity). Powdered materials were obtained from Sigma Chemical Co. (St. Louis, MO, USA). These powders were then solubilized in phosphate buffer solution at pH 7.0 and at normal room temperature. Several different dosages of the test compound up to and including 500 μg/mL were tested. It was found that 250 μg/mL generated the best outcome. For time course studies, the cells were treated with 10 μM finasteride or 250 μg/mL test compounds for 24 and 72 h. At the end of treatment, total cellular RNAs and lysates were prepared from these cells for analyses as detailed in the succeeding texts. All experiments were conducted in triplicate.

Real-time PCR. Total cellular RNAs were extracted using TRIzol reagent (Life Technologies). First-strand cDNAs were synthesized from total RNAs using MML-V reverse transcriptase (Life Technologies) and hexanucleotide random primers. Regular real-time PCR was performed using a Bio-Rad S1000 Thermal Cycler. Real-time PCR was carried out in a Roche 480 Real-Time PCR System, using SensiFAST SYBR No-Rox kits (Bioline). Beta-2 microglobulin served as the internal control. The amount of transcripts was calculated using the $2^{-\Delta\Delta Ct}$ formula (Bubner and Baldwin, 2004).

Western blot analysis. Cells were lysed in the Laemmli buffer. The lysate protein concentration was determined using Bio-Rad DC Protein Assays kit. Protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P membranes. Western blot analyses were carried out as previously described (Gilda and Gomes, 2015) using anti-5AR type 1 antibody (Santa Cruz Biotechnology). Secondary antibody was horseradish peroxidase-conjugated anti-IgG (Santa Cruz Biotechnology). Signals were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce). Internal loading control was β-actin, and the anti-beta-actin antibody was obtained from Sigma-Aldrich.

RESULTS

Inhibition of 5α-reductase and downstream genes in LNCaP cells

LNCaP cells are an immortalized androgen-sensitive human prostate adenocarcinoma cell line, originally derived from the left supraclavicular lymph node metastasis from a 50-year-old caucasian male in 1977. This cell line expresses numerous relevant markers, including those measured here. As expected, finasteride inhibited **5AR** type 1, 2, and 3 in LNCaP cells. We found that the test compound at 250 μg/mL was more potent and effective in blocking all three types of **5AR** expression than finasteride at 10 μM, which was the maximal dose that can be used to treat LNCaP cells without causing cytoxicity (Fig. 1). Similarly, western blot analysis showed that the test compound at 250 μg/mL suppressed type **5AR** protein expression more effectively than finasteride at 10 μM (Fig. 2). The western blot data validate that the inhibition occurred at both transcript and protein levels. We also examined that effect of these compounds on the expression of other

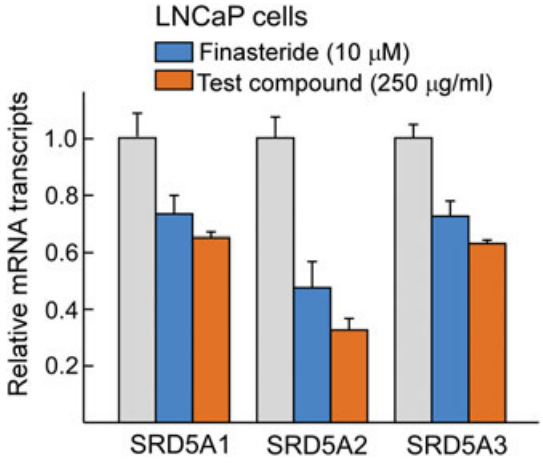

Figure 1. Effects of finasteride and test compound on 5-alpha reductase in LNCaP cells. LNCaP cells at confluence were treated with 10 μM finasteride or 250 μg/mL test compound for 24 h, and the mRNA transcript levels of 5-alpha reductase type 1, 2, and 3 (SRD5A1, SRD5A2, and SRD5A3) were quantified by real time (RT) PCR. Relative expression levels were determined against beta-2 microglobulin internal control. Therefore, at 250 μg/mL the test compound was more effective in blocking all three isoforms of 5AR, as compared with 10 μM (the maximum non-cytotoxic dose) of finasteride. All data were conducted in triplicate.

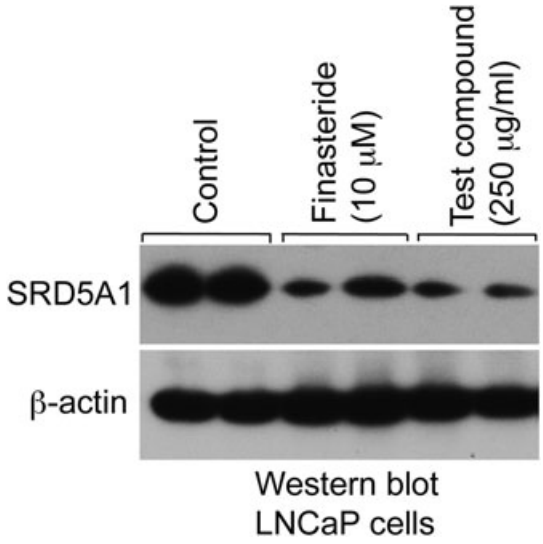

Figure 2. Inhibition of 5-alpha reductase protein in LNCaP cells. LNCaP cells at confluence were treated with 10 μM finasteride or 250 μg/mL test compound for 24 h, and SRD5A1 protein levels were analyzed by western blotting. Beta actin served as internal loading controls. Here, the test compound, at 250 μg/mL outperformed finasteride at its maximum non-cytotoxic dose of 10 μM, and showed that the inhibition occurred at the level of protein expression, and not solely at the level of gene transcription.

genes implicated in the pathogenesis of AGA, including dikkopf-1 (DKK-1), fibroblast growth factor (FGF)-1, 17beta-hydroxysteroid dehydrogenase Type 3 (17β-HSD-3), and transforming growth factor beta 1 and 2 (TGF-β1 and 2). As shown in Fig. 3, finasteride (10 μM) treatment reduced these genes to various degrees (Fig. 3A), but the effect of the test compound (250 μg/mL) was more effective in these inhibitions (Fig. 3B).

Inhibition of 5α-reductase and downstream genes in primary human follicle dermal papilla cells

We further investigated the effect of the test compound in primary human follicle dermal papilla cells. As shown in Fig. 4, finasteride at 10 μM suppressed the mRNA expression of 5AR type 1, 2, and 3, DKK-1, FGF-1, 17β-HSD-3, and TGF-β1 and β2 to various degrees in these cells (Fig. 4A), and the test compound at 250 μg/mL showed more potent inhibitory effects on these genes (Fig. 4B). Western blot showed that the test compound also markedly inhibited 5AR type 1 protein expression, thereby once again confirming the inhibition at the levels of transcription and protein translation (Fig. 5).

DISCUSSION

Both BPH and AGA are considered complex trait disorders, resulting from the expression of multiple genes interacting with epigenetic, environmental, biochemical, and temporal factors. While the blockade of 5AR remains a viable treatment approach, the role of inflammation constitutes a potentially intriguing therapeutic target. Epidemiological studies suggest that chronic prostatic inflammation appears to play a crucial role in BPH pathogenesis and progression (Ficarra *et al.*, 2014). Intriguingly, sustained microscopic follicular inflammation

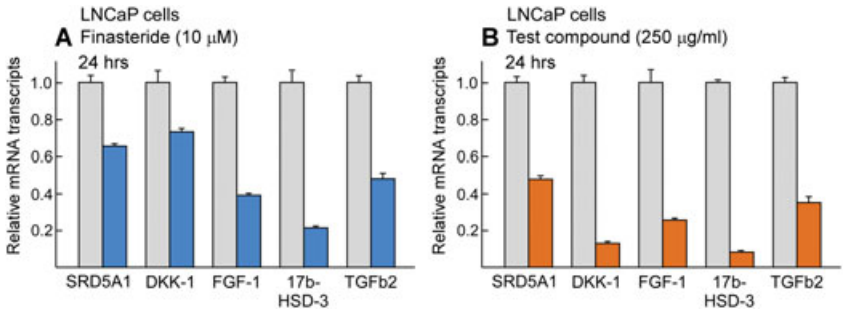

Figure 3. Inhibition of downstream gene expression in LNCaP cells. LNCaP cells at confluence were treated with 10 μM finasteride or 250 μg/mL test compound for 24 h, and the mRNA transcript levels of SRD5A1, DKK-1, FGF-1, 17b-HSD-3, and TGF β2 were assessed by real time RT-PCR. Relative expression levels were determined against beta-2 microglobulin internal control. Once again, at 250 μg/mL the test compound demonstrated greater reduction of transcript levels in tested markers than finasteride, in its maximum non-cytotoxic dose of 10 μM. All data were conducted in triplicate.

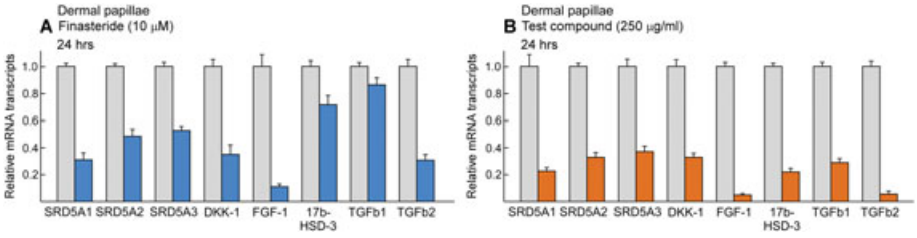

Figure 4. Effects of finasteride and test compound on gene expressions in human primary follicle dermal papilla cells. The cells were treated with 10 μM finasteride or 250 μg/mL test compound for 24 h, and the mRNA transcript levels of SRD5A1, SRD5A2, SRD5A3, DKK-1, FGF-1, 17b-HSD-3, TGF β1, and TGF β2 were quantified by real time RT-PCR. Relative expression levels were determined against beta-2 microglobulin internal control. All data were conducted in triplicate.

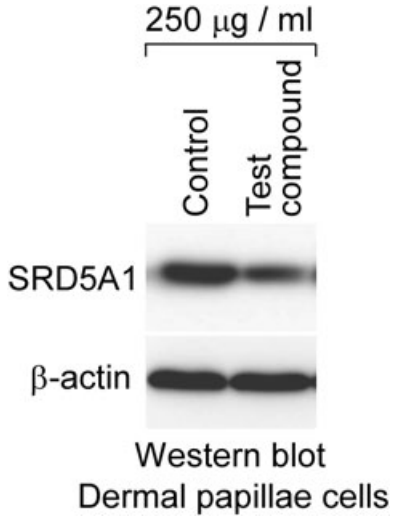

Figure 5. Inhibition of 5-alpha reductase protein by the test compound in human primary follicle dermal papilla cells. The cells were treated with 250 μg/mL test compound and SRD5A1 protein levels were analyzed by western blotting. Here, once again, at 250 μg/mL the test compound demonstrated greater inhibition at the level of protein expression, as compared with finasteride, in its maximum non-cytotoxic dose of 10 μM.

with connective tissue remodeling, eventually resulting in permanent hair loss, is now considered a cofactor in the complex etiology of AGA (Upton *et al.*, 2015). The development of safe and widely effective treatments for both disorders remains an elusive goal.

It is estimated that approximately 25% of modern drugs and as many as 60% of antineoplastic agents are derived from botanical sources (Abera, 2014). According to the World Health Organization, between 65% and 80% of the populations of developing countries currently use medicinal plants as remedies (Butterweck and Nahrstedt, 2012). The investigation of botanical source matter holds great potential as it is estimated that of the 300 000 plant species that exist in the world, only 15% have been evaluated to determine their pharmacological utility (Rivera *et al.*, 2014). A single botanical extraction may contain a complex repertoire of molecules that belong to a variety of chemical classes. This may also explain the frequently observed pleiotropic bioactivity spectra of such compounds, as well as the novel therapeutic opportunities that botanically based substances possess.

Previously, our group demonstrated the efficacy of naturally derived 5AR inhibitors incorporating stigmasterol and beta sitosterol in a series of placebo controlled and open-label clinical trials of men with AGA (Prager *et al.*, 2002). Subsequently, we extended our proprietary formulation by incorporating carnitine and thiocitic acid, which in addition to blocking 5-alpha reductase, also exhibited antiinflammatory characteristics in a well-established *in vitro* assay representing hair follicle keratinocyte gene expression.

In the present study, we tested a new version of our novel compound using a cell culture system that models prostate gene expression as well as one that models scalp hair follicle gene expression, with finasteride acting as a positive control. Following our guiding rationale, which posits 5 alpha-DHT acting in concert with downstream inflammation as a driving force in BPH and AGA, we interrogated the ability of our proprietary naturally derived compound to modulate potentially relevant gene markers in both disorders.

Here, we measured the differential activity of our test compound, and specifically its ability to inhibit 5AR in prostate LNCaP cells as well as primary human hair follicle cells derived from dermal papillae, at both transcript and protein levels. We chose to interrogate the LNCaP cell phenotype as a result of its well-described androgen sensitivity (Schuurmans *et al.*, 1988). We used both quantitative PCR and Western blot techniques to validate that the test compound is able to suppress both mRNA transcription and protein translation of 5AR in these cells. Having proved the translation-inhibiting activity of the test compound, we focused only on the transcript inhibition for other genes. Additionally, in both cell types we measured changes in mRNA transcript levels of the downstream genes: FGF-1, IL-6, DKK-1, TGF β1, TGF β2, and 17bHSD-3. Our data show that the test compound inhibits the expression of these genes in both cell systems, which provides a molecular basis to explain the therapeutic activities of the test compound in both BPH and AGA.

We chose the markers interrogated in this set of experiments, in part, because they are reflected in normal hair follicle homeostasis and appear to be disrupted in AGA. For example, animal model studies reveal that DKK-1, a powerful suppressor of the Wnt/β-catenin signaling pathway, induces anagen-to-catagen transition. DKK1 is one of the most upregulated genes in androgen-potentiated balding, with DKK-1 mRNA upregulated within hours after DHT treatment of hair follicles at the dermal papilla *in vitro* (Lei *et al.*, 2014). Likewise, TGF β has also been shown to influence scalp hair cycling and homeostasis (Hou *et al.*, 2015). Recent published work suggests a direct role for inflammatory stress in the pathogenesis of AGA in relation to cell senescence and migration as well as secretion of known hair follicle morphogenesis-inhibitory factors (Magro *et al.*, 2011).

Based on numerous well-controlled studies, it now seems highly probable that androgen sensitivity coupled with inflammatory stress contribute to the progression of BPH and AGA. Thus, the blockade of 5AR, coupled with the simultaneous suppression of downstream inflammatory stimuli appears to constitute a rational therapeutic approach in both BPH and AGA. Because of the lower risk of negative side effect and the potential ability to target multiple pathogenic pathways concomitantly, the use of naturally based pleiotropic compounds may offer an intriguing alternative to mainstream prescription drug monotherapy. The work reported here adds credibility to this hypothesis.

CONCLUSIONS

In summation, these experiments provide proof-of-concept *in vitro* evidence that novel, naturally based compositions targeting both 5AR and downstream inflammatory mediators may represent a promising strategy for the development of new treatments against

AGA and BPH, both of which represent disorders with significant unmet medical need in the aging population.

Acknowledgements

The authors are grateful for the technical support and advice from Best Life Pharmanaturals, Chandler, AZ. In particular, they thank Mr. Paul Finder for his company's expertise in formulating complex delivery systems. They also thank Elanna Romey and Vicki Weaver at Omega Tech Laboratories for their participation in preparation of the experimental protocols.

Conflict of Interest

Geno Marcovici is an officer of Advanced Restoration Technologies, Inc., the sponsor of this study. The other authors declare that they have no competing interests.

REFERENCES

Abera B. 2014. Medicinal plants used in traditional medicine by Oromo people, Ghimbi District, Southwest Ethiopia. *J Ethnobiol Ethnomed* **10**: 40.

Bubner B, Baldwin IT. 2004. Use of real-time PCR for determining copy number and zygosity in transgenic plants. *Plant Cell Rep* **23**(5): 263–71.

Butterweck V, Nahrstedt A. 2012. What is the best strategy for preclinical testing of botanicals? A critical perspective. *Planta Med* **78**(8): 747–54.

Chen W, Zouboulis CC, Orfanos CE. 1996. The 5 alpha-reductase system and its inhibitors. Recent development and its perspective in treating androgen-dependent skin disorders. *Dermatology* **193**(3): 177–84.

Chittur S, Parr B, Marcovici G. 2011. Inhibition of inflammatory gene expression in keratinocytes using a composition containing carnitine, thioctic Acid and saw palmetto extract. *Evid Based Complement Alternat Med* **2011**: 985345: . DOI: http://dx.doi.org/10.1093/ecam/nep102.

Corona G, Vignozzi L, Rastrelli G, Lotti F, Cipriani S, Maggi M. 2014. Benign prostatic hyperplasia: a new metabolic disease of the aging male and its correlation with sexual dysfunctions. *Int J Endocrinol* **2014**(2014*:* 329456): .

Ficarra V, Rossanese M, Zazzara M, *et al.* 2014. The role of inflammation in lower urinary tract symptoms (LUTS) due to benign prostatic hyperplasia (BPH) and its potential impact on medical therapy. *Curr Urol Rep* **15**(12): 463. DOI:http://dx.doi.org/10.1007/s11934-014-0463-9.

Gilda JE, Gomes AV. 2015. Western blotting using in-gel protein labeling as a normalization control: stain-free technology. *Methods Mol Biol* **1295**: 381–91.

Hou C, Miao Y, Wang J, Wang X, Chen CY, Hu ZQ. 2015. Collagenase IV plays an important role in regulating hair cycle by inducing VEGF, IGF-1, andTGF-β expression. *Drug Des Devel Ther* **9**: 5373–83.

Kumar VL, Wahane VD. 2008. Current status of 5alpha-reductase inhibitors in the treatment of benign hyperplasia of prostate. *Indian J Med Sci* **62**(4): 167–75.

Lee SH, Lee JY. 2014. Current role of treatment in men with lower urinary tract symptoms combined with overactive bladder. *Prostate Int* **2**(2): 43–9.

Lei M, Guo H, Qiu W, *et al.* 2014. Modulating hair follicle size with Wnt10b/DKK1 during hair regeneration. *Exp Dermatol* **23**(6): 407–13.

Magro CM, Rossi A, Poe J, Manhas-Bhutani S, Sadick N. 2011. The role of inflammation and immunity in the pathogenesis of androgenetic alopecia. *J Drugs Dermatol* **10**(12): 1404–11.

McElwee KJ, Shapiro JS. 2012. Promising therapies for treating and/or preventing androgenic alopecia. *Skin Therapy Lett* **17**(6): 1–4.

Prager N, Bickett K, French N, Marcovici G. 2002. A randomized, double-blind, placebo-controlled trial to determine the effectiveness of botanically derived inhibitors of 5-alpha-reductase in the treatment of androgenetic alopecia. *J Altern Complement Med* **8**(2): 143–52.

Radomski SB. 2014. Update on medical therapy for male LUTS. *Can Urol Assoc J* **8**(7-8 Suppl 5): S148–50.

Rivera D, Allkin R, Obón C, Alcaraz F, Verpoorte R, Heinrich M. 2014. What is in a name? The need for accurate scientific nomenclature for plants. *J Ethnopharmacol* **152**(3): 393–402.

Schuurmans AL, Bolt J, Mulder E. 1988. Androgens stimulate both growth rate and epidermal growth factor receptor activity of the human prostate tumor cell LNCaP. *Prostate* **12**(1): 55–63.

Traish AM, Mulgaonkar A, Giordano N. 2014. The dark side of 5α-reductase inhibitors' therapy: sexual dysfunction, high Gleason grade prostate cancer and depression. *Korean J Urol* **55**(6): 367–79.

Upton JH, Hannen RF, Bahta AW, Farjo N, Farjo B, Philpott MP. 2015. Oxidative stress-associated senescence in dermal papilla cells of men with androgenetic alopecia. *J Invest Dermatol* **135**(5): 1244–52.

Vujovic A, Del Marmo V. 2014. The female pattern hair loss: review of etiopathogenesis and diagnosis. *Biomed Res Int* **2014***:* 767628: .

Wroński S. 2014. The new horizons of pharmacotherapy. Unexpected pharmacological actions and a new therapeutic strategy of phosphodiesterase-5 inhibitors. *Cent European J Urol* **67**(3): 314–8.

Yoon JH, Baek SJ. 2005. Molecular targets of dietary polyphenols with anti- inflammatory properties. *Yonsei Med J* **46**(5): 585–596.

Young JW, Conte ET, Leavitt ML, Nafz MA, Schroeter AL. 1991. Cutaneous immunopathology of androgenetic alopecia. *J Am Osteopath Assoc* **91**(8): 765–771.

