BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 21(1), 49-53 (2013)

Finasteride Increases the Expression of Hemoxygenase-1 (HO-1) and NF-E2-Related Factor-2 (Nrf2) Proteins in PC-3 Cells: Implication of Finasteride-Mediated High-Grade Prostate Tumor Occurrence

Do-Kyung Yun[1], June Lee[2] and Young-Sam Keum[1,*]

[1]Department of Biochemistry, College of Pharmacy, Dongguk University, Goyang 410-820,
[2]Department of Chemistry, Dongguk University, Seoul 100-715, Republic of Korea

Abstract

A number of naturally-occurring or synthetic chemicals have been reported to exhibit prostate chemopreventive effects. Synthetic 5α-reductase (5-AR) inhibitors, e.g. finasteride and durasteride, gained special interests as possible prostate chemopreventive agents. Indeed, two large-scale epidemiological studies have demonstrated that finasteride or durasteride significantly reduced the incidence of prostate cancer formation in men. However, these studies have raised an unexpected concern; finasteride and durasteride increased the occurrence of aggressive prostate tumor formation. In the present study, we have observed that treatment of finasteride did not affect the growth of androgen-refractory PC-3 prostate cancer cells. Finasteride also failed to induce apoptosis or affect the expression of proto-oncogenes in PC-3 cells. Interestingly, we found that treatment of finasteride induced the expression of Nrf2 and HO-1 proteins in PC-3 cells. In particular, basal level of Nrf2 protein was higher in androgen-refractory prostate cancer cells, e.g. DU-145 and PC-3 cells, compared with androgen-responsive prostate cancer cells, e.g. LNCaP cells. Also, treatment of finasteride resulted in a selective induction of Nrf2 protein in DU-145 and PC-3 cells, but not in LNCaP cells. In view of the fact that upregulation of Nrf2-mediated phase II cytoprotective enzymes contribute to attenuating tumor promotion in normal cells, but, on the other hand, confers a selective advantage for cancer cells to proliferate and survive against chemical carcinogenesis and other forms of toxicity, we propose that finasteride-mediated induction of Nrf2 protein might be responsible, at least in part, for an increased risk of high-grade prostate tumor formation in men.

Key Words: Chemoprevention, Finasteride, 5α-reductase (5-AR), NF-E2-related factor-2 (Nrf2)

INTRODUCTION

5α-reductase (5-AR) enzyme is responsible for a metabolic conversion of testosterone (T) into more active androgen hormone, dihydrotestosterone (DHT) (Gormley *et al*., 1992). Although T is the most abundant serum androgen, DHT is the main prostatic androgen and exhibits a stronger binding affinity towards androgen receptor (AR). Finasteride, a 4-aza-steroid and analogue of testosterone, works by acting as a potent and specific, competitive inhibitor of one of the two subtypes of 5-AR, specifically the type II isozyme (Aggarwal *et al*., 2010). Finasteride has been sold under the brand name of Proscar® or Propecia® for the treatment of benign prostatic hyperplasia (BPH) and androgenic alopecia, respectively (Rittmaster, 1997). In addition, finasteride has been tried alone or in combination with other drugs, including non-steroidal anti-androgens in patients, who underwent a therapeutic failure of pre-radical prostatectomy (RP) or radiotherapy (Gooren, 2005). Because finasteride and durasteride are currently on the market for treatment of BPH, it was assumed that the potential of 5-AR chemical inhibitors would be able to serve as putative prostate chemopreventive agents.

Chemoprevention refers to an active clinical strategy to find out natural or synthetic chemical agents that can inhibit, delay, or even reverse the carcinogenesis in human (Surh, 2003). Because prostate cancer is among the most common types of cancer in men, finding out effective prostate chemopreventive agent(s) would have a profound clinical significance. Three large-scale prostate chemopreventive clinical trials have been conducted, by far, to examine the prostate cancer-preventive

Open Access **http://dx.doi.org/10.4062/biomolther.2012.080**

Received Oct 15, 2012 **Revised** Dec 8, 2012 **Accepted** Dec 11, 2012

***Corresponding Author**
E-mail: keum03@dongguk.edu
Tel: +82-31-961-5215, Fax: +82-31-961-5206
The first two authors contributed equally to this work.

effects of selected natural or synthetic agents: (1) Selenium and Vitamin E Cancer Prevention Trials (SELECT) (Lippman *et al*., 2009), (2) Prostate Cancer Prevention Trial (PCPT) (Thompson *et al*., 2003), and (3) Reduction by Durasteride of Prostate Cancer Events (REDUCE) (Andriole *et al*., 2010). Although natural selenium and vitamin E have been shown to exhibit significant prostate chemopreventive effects in various preclinical animal models, the SELECT trial showed discouraging results that either selenium or vitamin E failed to reduce the incidence of prostate cancer in men (Lippman *et al*., 2009). Contrary to the SELECT clinical trial, the PCPT and REDUCE clinical trials yielded a promise in prostate cancer prevention research. These two clinical trials were conducted to examine the prostate chemopreventive effects of two synthetic 5α-reductase (5-AR) inhibitors, e.g. finasteride (for the PCPT) and durasteride (for the REDUCE). Indeed, the results of PCPT clinical trial showed that men given with finasteride had a clear reduction (23%) in prostate cancer incidence after 7 years, when compared with those given with a placebo (Thompson *et al*., 2003). Likewise, the REDUCE clinical trial showed an analogous finding that the prostate cancer incidence in men was significantly reduced (25%) after 4 years, when the subjects consumed durasteride on a regular basis (Andriole *et al*., 2010). However, both the PCPT and REDUCE trials raised a common and unexpected concern: the occurrence of aggressive types of prostate tumors (Gleason scores 7-10) was elevated in the subjects given with finasteride or durasteride, compared with those given with placebo (Azzouni and Mohler, 2012). This fact makes it still inappropriate to prescribe finasteride or durasteride as prostate chemopreventive agents in the clinical setting (Walsh, 2010). Therefore, it seems necessary at present to understand why 5-AR inhibitors promote the occurrence of aggressive prostate cancers in human (Nacusi and Tindall, 2011). Our study was initiated to address this issue.

MATERIALS AND METHODS

Cell culture, chemicals, and antibody

LNCaP, DU-145, PC-3, and U2OS cells were purchased from Korean Cell Line Bank (Seoul, Republic of Korea) and cultured at 37°C and 5% CO_2 in Dulbelco's Minimal Essential Medium (DMEM), supplemented with 10% FBS, 2.2 mg/ml sodium bicarbonate, 100 units/ml penicillin, and 100 units/ml streptomycin. All chemicals used in the study, including finasteride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal Nrf2, Keap1 and total actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The other antibodies, used in the present study were purchased from Cell Signaling Technologies (Danvers, MA, USA).

Trypan-blue exclusion assay

PC-3 cells were seeded in 6-well plates at a density of 1×10^5 per well. Following an exposure to finasteride for 24 h and 48 h, cells were collected by trypsinization, followed by centrifugation at 1,000 *g* for 5 min. Collected cells were rinsed with ice-cold phosphate-buffer saline (PBS) solution (pH 7.4) 3 times and mixed with 100 μl of PBS together with an equal amount of 0.4% trypan blue reagent. After counting viable cell numbers that excluded trypan blue reagent by hemacytometer, total number of viable cells was calculated by doubling a dilution factor (×2).

Western blot analysis

For preparation of whole cell lysates, cells were harvested in whole cell lysis buffer [10 mmol/L Tris-HCl (pH 7.9), 250 mmol/L NaCl, 30 mmol/L sodium bisphosphate, 50 mmol/L sodium fluoride, 0.5% Triton X-100, 10% glycerol, 1×proteinase inhibitor mixture,] for 30 min on ice. Lysates were then collected by centrifugation at 14,800 *g* for 30 min. Protein concentrations were determined by the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Aliquots of supernatant, containing 30 μg proteins were boiled in 1× SDS sample loading buffer for 2 min and resolved using 12% SDS-PAGE. Proteins in SDS-polyacrylamide gel were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% fat-free milk in PBS-Tween 20 (PBST, 0.1% Tween 20) at room temperature for 2 h. The membrane was then probed with primary antibodies (1:1,000) in PBS overnight at 4°C. Blots were rinsed with PBST (PBS with 0.1% Tween-20) three times and then incubated with 1:5,000 dilution of horseradish peroxidase–conjugated second antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. The blots were washed in PBST buffer for 5 min thee times and the transferred protein was visualized, using the enhanced chemiiluminescence (ECL).

Measurement of dual luciferase activity

U2OS cells were plated in six-well plates and allowed to grow around 70% confluency. 0.1 μg COX-2-, MMP2- and NF-κB-promoter-driven firefly luciferase constructs were co-transfected with 0.1 μg *Renilla* luciferase plasmid, using lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). After transfection, cells were treated with DMSO or finasteride for additional 48 h. Cells were then collected and the dual luciferase activity was measured by the GLOMAX Multi-detection system (Promega, Madison, WI, USA). The measured firefly luciferase activity was normalized against the measured *Renilla* luciferase activity and the resulting value was expressed as a fold induction over the control. Values are expressed as mean ± SD of experiments and statistical analysis was performed, using Student *t*-test with n=6.

RESULTS AND DISCUSSION

We have attempted to find out the correlative biomarker(s) that might account for the clinical observation how an intake of 5-AR inhibitors could result in an increased incidence of high-grade prostate tumors in men. PC-3 cells were chosen in the

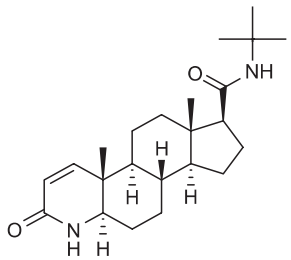

Fig. 1. Chemical structure of finasteride.

present study, because they exhibit two biological characteristics of highly aggressive prostate cancers: (1) a lack of p53 tumor suppressor protein and (2) an androgen-independent growth (Aalinkeel *et al*., 2004). First, we exposed PC-3 cells to finasteride (Fig. 1) for 24 h and 48 h and examined whether finasteride might exert cytotoxic effects on the growth of PC-3 cells. After finasteride treatment, PC-3 cells were collected by trypsinization and the live cell number was counted by the trypan-blue exclusion assay. It should be noted that we were unable to employ durasteride (a potent inhibitor with a dual specificity against both type-1 and type-2 5-AR enzymes) in our study, because it is commercially unavailable due to a patent issue (Avodart®, GlaxoSmithKlein, USA). Our results show that finasteride failed to exhibit inhibitory effects on the growth of PC-3 cells (Fig. 2A). In contrast, treatment of etoposide, a selective chemical inhibitor of topoisomerase II enzyme and a positive control compound for apoptotic inducer, resulted in a significant cell death at 48h post-treatment (Fig. 2A). Western blot analysis supports for this observation: an exposure of finasteride to PC-3 cells did not induce a cleavage of Caspase-3, Caspase-7 and PARP proteins (Fig. 2B). However, we observed that a cleavage of Caspase-9 protein occurred after finasteride treatment (Fig. 2B). Although it is unclear how finasteride induced activation of Caspase-9 protein in PC-3 cells, we conclude that finasteride does not induce apoptosis in PC-3 cells because a cleavage of Caspase-9 protein is an upstream apoptotic event, compared with a cleavage of Caspase-3 and PARP proteins (Riedl and Shi, 2004). It is well accepted that activation of proto-oncogene is responsible for a malignant transformation of prostate cancer. In this context, we examined whether treatment of finasteride could affect the protein expression of proto-oncogenes in PC-3 cells. Western blot analysis shows that treatment of finasteride didn't affect the expression of c-Jun, c-Raf, c-Rel, c-Abl and Src proteins (Fig. 3A), the expression of which is generally increased in the malignant type of prostate cancer. In addition, we observed that an exposure of finasteride did not affect the COX-2-, MMP2- and NF-κB-promoter-driven luciferase activities (Fig. 3B). Based on these observations, we conclude that the induction of proto-oncogenes is not responsible for an increased incidence of high-grade prostate tumor formation by finasteride.

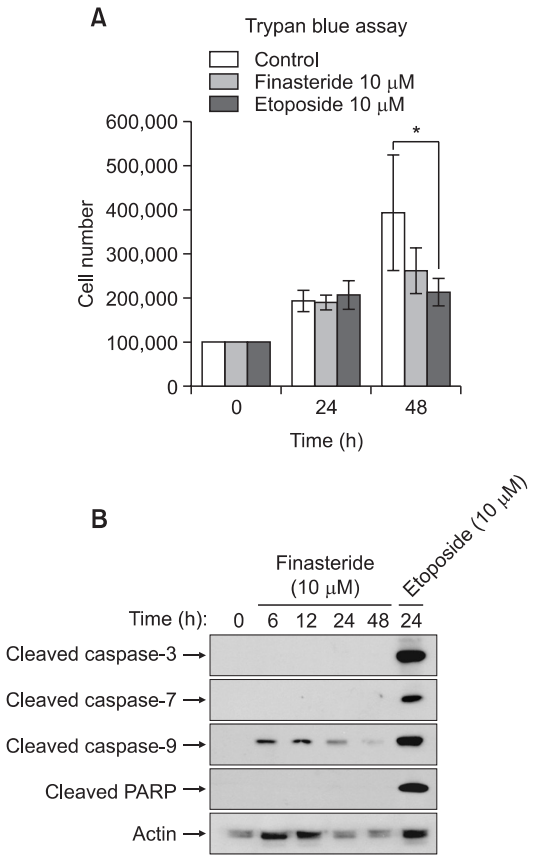

Fig. 2. Effect of finasteride on the viability and apoptotic markers of PC-3 cells. (A) The viability of PC-3 cells was determined by trypan-exclusion assay as described in Materials and Methods. Each bar represents mean ± standard deviation (SD) with **p*<0.05 (n=6). (B) Expression of cleaved Caspase-3, -7, -9 and PARP after finasteride treatment was determined by Western blot analysis. PC-3 cell lysates, exposed to etoposide were used as positive control of the Western blot experiment and total actin blot shows a loading of equal amounts of sample in each wells.

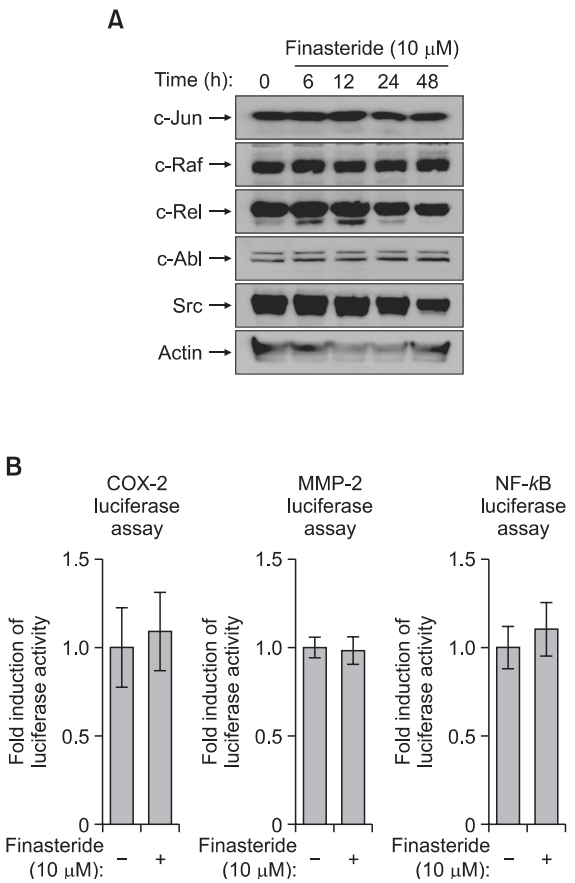

Fig. 3. Effect of finasteride on the expression of proto-oncogene expression in PC-3 cells. (A) Expression changes of selected proto-oncogenes: c-Jun, c-Raf, c-Rel, c-Abl, Src in response to finasteride were examined by Western blot analysis. Actin blot illustrates an equal loading of samples in each well. (B) The effect of finasteride on COX-2-, MMP2- and NF-κB-luciferase activity was measured by the dual-luciferase activity.

The NF-E2-related factor-2 (Nrf2) is a transcriptional factor that belongs to the Cap'n'Collar (CNC) subfamilty of basic leucine-zipper (bZIP) family of transcriptional factors (Keum, 2011). Nrf2 is constantly degraded in the cytoplasm under basal conditions, but, upon exposure to oxidants and eletrophiles, degradation of Nrf2 protein is halted, which makes it stabilized and free to translocate into the nucleus, thereby activating target genes by binding to the cis-acting element in the genome, termed antioxidant response element (ARE). Nrf2 is a master transcription factor that is involved in oxidative and xenobiotic stress responses by transcriptionally activating phase II cytoprotective genes, such as hemeoxygenase-1 (HO-1), NAD[P]H:quinone oxidoreductases (NQO1) and glutathione S-transferase (GST) (Tong *et al*., 2006). The cellular activity and stability of Nrf2 are regulated by another cytosolic E3 ligase protein, e.g. Kelch-like ECH-associated protein 1 (Keap1) through a variety of intracellular signaling kinases, including MAPKs (mitogen-activated protein kinases), PI3K (phosphatidylinositol 3-kinase), PKC (protein kinase C), and CK2 (Casein kinase 2) (Keum *et al*., 2004). Because the activation of Nrf2-mediated signaling pathway is an adaptive response that contributes to detoxifying chemical carcinogens and electrophiles in response to a variety of endogenous and exogenous stresses, it has been thought that boosting up the Nrf2 activity is regarded as a feasible way of clinical chemopreventive strategy. Paradoxically, accumulating evidence also indicates that Nrf2 is often mutated in several types of human cancer and such mutation contributes to a constant activation of Nrf2-dependent phase II cytoprotective genes in cancer. This fact suggests that, unlike in normal tissues, an aberrant activation of Nrf2-dependent dependent gene expression contributes to promoting cell survival in cancer (Kensler and Wakabayashi, 2010).

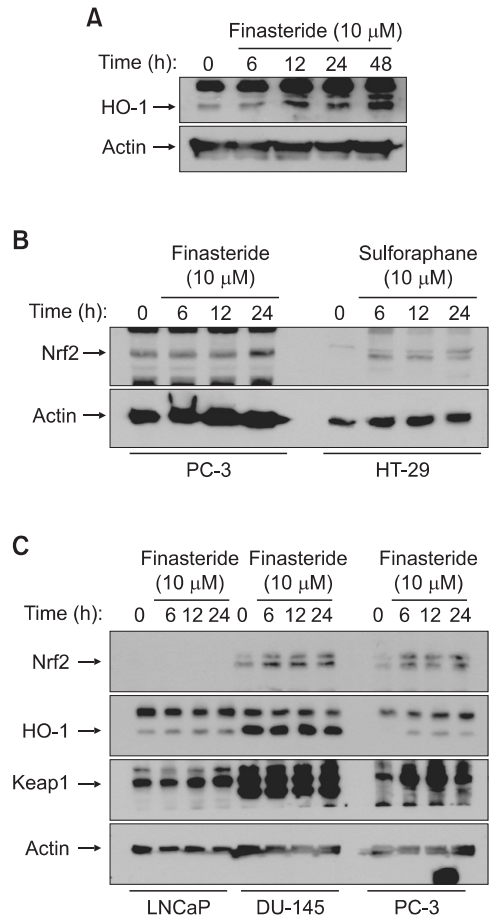

Fig. 4. Finasteride induces the expression of HO-1 and Nrf2 proteins in PC-3 cells. (A) Effect of finasteride on the expression of HO-1 protein in PC-3 cells. (B) Effect of finasteride on the expression of Nrf2 protein in PC-3 cells. Cell lysates showing the induction of Nrf2 protein by sulforaphane in HT-29 cells were loaded as a positive control. (C) Finasteride induces the expression of Nrf2 protein in DU-145 and PC-3 cells, but not in LNCaP cells. The cell lysates were loaded and Western blot analysis was conducted, using HO-1, Keap1 and actin polyclonal antibodies.

Our result shows that an exposure of finasteride to PC-3 cells increased the expression of HO-1 protein in a time-dependent manner (Fig. 4A), whose transcriptional expression is primarily mediated by Nrf2 (Paine *et al*., 2010). Likewise, the expression of Nrf2 protein in PC-3 cells was increased in response to finasteride treatment (Fig. 4B). Finally, we observed that the basal level of Nrf2 protein is elevated in androgen-refractory prostate cells, e.g. DU-145 and PC-3 cells, compared with androgen-responsive prostate cells, e.g. LNCaP cells and finasteride treatment significantly induced the expression of Nrf2 protein in DU-145 and PC-3 cells, but not in LNCaP cells (Fig. 4C). It is also interesting that the finasteride-mediated induction of HO-1 protein was observed in PC-3 cells, but not in LNCaP and DU-145 cells and the expression of Keap1 protein was unaffected in all tested cell lines (Fig. 4C). It is unclear at present why the induction of Nrf2 protein is not translated to the induction of HO-1 protein in DU-145 cells, we presume that Nrf2-mediated induction of HO-1 might require the existence of p53 protein that is present in PC-3 cells, but absent in DU-145 cells. Together, our data suggests that the induction of Nrf2 protein by finasteride occurs exclusively in androgen-refractory prostate cells and a high abundance together with a selective upregulation of Nrf2 protein by finasteride might contribute to the survival of androgen-refractory prostate cancers through the transcriptional activation of phase II cytoprotective enzymes, including HO-1. On the other hand, it is interesting that some scientists dispute the observation of an increased incidence of aggressive prostate tumors in patients, given with a finasteride or durasteride. They suggest that observing an increased incidence of high-grade prostate tumor in the finasteride or durasteride arm, compared with the placebo arm in the PCPT and REDUCE clinical trials can be ascribed to a bias rather to a change in disease biology, such as (1) the sensitivity of prostate-specific antigen, (2) a disproportionate sampling of the patient prostate gland by random needle biopsy, and (3) an inappropriate statistical analysis (Bostwick *et al*., 2004). Although further studies are required to address these issues, we believe that a selective induction of Nrf2 protein by finasteride may aid in understanding why finasteride promotes the malignancy of prostate cancer in the clinical setting, if the interpretation of the above clinical studies still holds.

ACKNOWLEDGMENTS

This work was supported by the GRRC program of Gyeonggi province [(GRRC-DONGGUK2012-A01), Study of control of viral diseases].

REFERENCES

Aalinkeel, R., Nair, M. P., Sufrin, G., Mahajan, S. D., Chadha, K. C., Chawda, R. P. and Schwartz, S. A. (2004) Gene expression of angiogenic factors correlates with metastatic potential of prostate cancer cells. *Cancer Res.* **64**, 5311-5321.

Aggarwal, S., Thareja, S., Verma, A., Bhardwaj, T. R. and Kumar, M. (2010) An overview on 5alpha-reductase inhibitors. *Steroids* **75**, 109-153.

Andriole, G. L., Bostwick, D. G., Brawley, O. W., Gomella, L. G., Marberger, M., Montorsi, F., Pettaway, C. A., Tammela, T. L., Teloken, C., Tindall, D. J., Somerville, M. C., Wilson, T. H., Fowler, I. L. and Rittmaster, R. S. (2010) Effect of dutasteride on the risk of prostate cancer. *N. Engl. J. Med.* **362**, 1192-1202.

Azzouni, F. and Mohler, J. (2012) Role of 5α-reductase inhibitors in prostate cancer prevention and treatment. *Urology* **79**, 1197-1205.

Bostwick, D. G., Qian, J., Civantos, F., Roehrborn, C. G. and Montironi, R. (2004) Does finasteride alter the pathology of the prostate and cancer grading? *Clin. Prostate Cancer* **2**, 228-235.

Gooren, L. (2005) Hormone treatment of the adult transsexual patient. *Horm. Res.* **64 Suppl 2**, 31-36.

Gormley, G. J., Stoner, E., Bruskewitz, R. C., Imperato-McGinley, J., Walsh, P. C., McConnell, J. D., Andriole, G. L., Geller, J., Bracken, B. R., Tenover, J. S. and *et al.* (1992) The effect of finasteride in men with benign prostatic hyperplasia. The Finasteride Study Group. *N. Engl. J. Med.* **327**, 1185-1191.

Kensler, T. W. and Wakabayashi, N. (2010) Nrf2: friend or foe for chemoprevention? *Carcinogenesis* **31**, 90-99.

Keum, Y. S. (2011) Regulation of the Keap1/Nrf2 system by chemopreventive sulforaphane: implications of posttranslational modifications. *Ann N Y Acad Sci* **1229**, 184-189.

Keum, Y. S., Jeong, W. S. and Kong, A. N. (2004) Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms. *Mutat. Res.* **555**, 191-202.

Lippman, S. M., Klein, E. A., Goodman, P. J., Lucia, M. S., Thompson, I. M., Ford, L. G., Parnes, H. L., Minasian, L. M., Gaziano, J. M., Hartline, J. A., Parsons, J. K., Bearden, J. D. 3rd, Crawford, E. D., Goodman, G. E., Claudio, J., Winquist, E., Cook, E. D., Karp, D. D., Walther, P., Lieber, M. M., Kristal, A. R., Darke, A. K., Arnold, K. B., Ganz, P. A., Santella, R. M., Albanes, D., Taylor, P. R., Probstfield, J. L., Jagpal, T. J., Crowley, J. J., Meyskens, F. L. Jr., Baker, L. H. and Coltman, C. A. Jr. (2009) Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA* **301**, 39-51.

Nacusi, L. P. and Tindall, D. J. (2011) Targeting 5α-reductase for prostate cancer prevention and treatment. *Nat. Rev. Urol.* **8**, 378-384.

Paine, A., Eiz-Vesper, B., Blasczyk, R. and Immenschuh, S. (2010) Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. *Biochem. Pharmacol.* **80**, 1895-1903.

Riedl, S. J. and Shi, Y. (2004) Molecular mechanisms of caspase regulation during apoptosis. *Nat. Rev. Mol. Cell Biol.* **5**, 897-907.

Rittmaster, R. S. (1997) 5alpha-reductase inhibitors. *J. Androl.* **18**, 582-587.

Surh, Y. J. (2003) Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer* **3**, 768-780.

Thompson, I. M., Goodman, P. J., Tangen, C. M., Lucia, M. S., Miller, G. J., Ford, L. G., Lieber, M. M., Cespedes, R. D., Atkins, J. N., Lippman, S. M., Carlin, S. M., Ryan, A., Szczepanek, C. M., Crowley, J. J. and Coltman, C. A. Jr. (2003) The influence of finasteride on the development of prostate cancer. *N. Engl. J. Med.* **349**, 215-224.

Tong, K. I., Kobayashi, A., Katsuoka, F. and Yamamoto, M. (2006) Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. *Biol. Chem.* **387**, 1311-1320.

Walsh, P. C. (2010) Chemoprevention of prostate cancer. *N. Engl. J. Med.* **362**, 1237-1238.