



Inhibition of 5 α -reductase in the rat prostate by *Cimicifuga racemosa*

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Abstract

Objectives: Prostate cancers and many thereof derived cell lines, as the LNCaP cells, grow androgen-dependent. In vivo testosterone is locally converted by 5 α -reductase to 5 α -dihydrotestosterone (5 α -DHT) which is the major androgenic principle in prostates and seminal vesicles. The occurrence of prostate cancer and growth of LNCaP cells can be effectively inhibited by finasteride, a synthetic 5 α -reductase inhibitor and by a black cohosh (*Cimicifuga racemosa*, CR) extract. In the present contribution we tested whether the aqueous/ethanolic *C. racemosa* extract BNO 1055 contains 5 α -reductase inhibitors.

Methods: Immature 24-day-old male rats were fed with testosterone (T)-containing food and injected with 30 mg CR BNO 1055 or 0.5 mg finasteride for 5 days. Average daily T-uptake was 39 mg/animal. Other animals remained untreated or received vehicle injections only.

Results: In comparison to totally untreated rats the testosterone treatment increased weight of prostates and seminal vesicles 3–5-fold and this proliferation was largely and equipotently inhibited by finasteride and CR BNO 1055. 5 α -Dihydrotestosterone concentrations in prostate tissue extracts were also reduced by both compounds and the testosterone-upregulated androgen receptor and insulin like growth factor I gene expression inhibited in the seminal vesicles.

Conclusion: Taken together, these results indicate that the CR extract BNO 1055 contains one or more potent 5 α -reductase inhibitors which may make this extract suitable for the prevention and treatment of prostate cancer and possibly of benign prostate hyperplasia (BPH).

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1. Introduction

It is well accepted that prostates and seminal vesicles proliferate in response to high circulating testosterone (T) levels. The androgen T, however, does not act per se, but needs to be reduced to 5 α -dihydrotestosterone (5 α -DHT) by an enzyme called 5 α -reductase [1–3].

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An enzyme with identical functions though coded on a different gene, is also present in a number of cells in the organism including in fibroblasts. The prostate and seminal vesicles predominant reductase is called 5 α -reductase type 2, the other enzyme primarily expressed in fibroblasts, hair follicles, sebum glands is the 5 α -reductase 1. Inhibition of these two enzyme isoforms largely prevents testosterone effects in the prostate, seminal vesicles, on hair growth and sebum production [4–6]. The synthetic 5 α -reductase inhibitor finasteride inhibits both isoforms of this enzyme [7] and in long-term studies reduction of the incidence of prostate cancer has been reported under this reductase inhibitor [8–14]. Also the development of benign prostatic hyperplasia (BPH) appears to be inhibited by higher doses of 5 α -reductase inhibitors [7,15]. Inhibition of 5 α -reductase by finasteride had reportedly also inhibitory effects on sebum production and acne [4–6].

Recently we demonstrated that the aqueous/ethanolic extract of *Cimicifuga racemosa* named CR BNO 1055 inhibited proliferation of the human prostate cancer cell line LNCaP [16], and in preliminary experiments we had observed that treatment of male rats with this extract resulted in significantly lower prostate weights (unpublished) while no effect was seen in orchidectomized (orx) animals [17]. Therefore we speculated that one or more compound(s) in CR BNO 1055 may exert 5 α -reductase inhibiting properties. The aim of the present study was therefore to investigate in vivo the effects of the special extract BNO 1055 on the two androgen-stimulated organs, the prostate and the seminal vesicles and on intraprostatic 5 α -DHT concentrations and on genes in the seminal vesicles known to be regulated by androgens or estrogens. In the prostate it appears that not only 5 α -DHT regulate prostate cell proliferation through androgen receptors which are primarily located in epithelial cells [18] but also by estrogens which act via the newly cloned estrogen receptor β [19–22] which are primarily located in the same cell type [23]. It appears that estrogens acting via ER β have antiproliferative effects in the prostate [24].

In male rats prostates and seminal vesicles shrink following orchidectomy (orx) but can be stimulated by 5 α -reducible androgens and inhibition of 5 α -reductase results in a substantial reduction of prostatic proliferation [18,23]. This is the basis of the Hershberger assay, a test for androgenic and antiandrogenic activi-

ties. Under the working hypothesis that CR BNO 1055 inhibits 5 α -reductase we applied this assay. Instead of mature orchidectomized rats as originally proposed for the Hershberger assay [25,26] we used for the present study intact immature 24-day-old rats which were fed with testosterone and subcutaneously injected with the CR extract BNO 1055 or with finasteride as positive or the solvent as negative controls for 4 days. This animal model proved to be as sensitive as the original model. In addition, we measured 5 α -DHT concentrations in extracts of the animal prostates. Since seminal vesicles react to 5 α -DHT very similar as prostates we measured the mRNA transcripts of a two androgen-regulated genes in extracts of the seminal vesicles. The expression of the androgen receptor and of insulin like growth factor I is known to be regulated by androgens [27–29].

2. Materials and methods

The CR extract was obtained from Bionorica (Neumarkt, Germany) and was an aqueous/ethanolic extract of the rhizomes of cultivated plants [30]. In short: the preparation of the CR extract BNO 1055 was as follows: finely ground CR rhizomes were extracted with five times the amount of 50% (m/m) water/ethanol for 48 h with a percolation speed of 500 kg/h. After filtration the extract was concentrated under vacuum and evaporated to dryness at 100–140 mbar according to a patent-protected process. Product temperature did not exceed 40 °C.

The animal experiments were approved by the Bezirksregierung Braunschweig (Permission No. Az 509.42502/01-36.03 dated 13.10.2003).

Mature, 3-months-old male and female Sprague Dawley rats were purchased (Winkelmann, Borchen, Germany) and kept under soy-free potato protein-supplemented food for at least four weeks. They were then mated and pups were separated from their mothers at postnatal day 21. The male young animals were then also kept under soy-free potato protein-supplemented food until day 24 at which time the pelleted food was supplemented with 3 mg testosterone/g which resulted in slightly supraphysiologic serum T concentrations of approximately 8.5 ng/ml. Due to the irregular food intake of the animals these serum values varied largely. Animals in subgroups ($n=20$) were daily subcuta-

neously injected either with 25 mg of the CR extract BNO 1055 or with 0.5 mg finasteride for 4 days. These injections were given at 07:00 h in the morning; at the last day, 4–6 h after the last injection animals were decapitated, blood was collected from the trunk, the prostates and the seminal vesicles were collected and cleaned from fat tissue and weight. The prostates were homogenized and the homogenates were centrifuged at $10,000 \times g$ for 10 min and the supernatant used for the measurement of 5α -DHT by a commercially available radioimmunoassay (IBL, Hamburg, Germany) which crossreacted with testosterone to less than 8.3%. Four months later these experiments were repeated with offsprings of the same dams. Results were identical and therefore the data were lumped together.

The seminal vesicles were dissected and snap-frozen in liquid nitrogen within 3 min to minimize degradation of RNA. Samples were stored at -70°C until RNA preparation. Total RNA was extracted from the seminal vesicles. Tissue samples were chilled in liquid nitrogen and pulverized in a 5 ml teflon container with a tissue homogenizer (Micro-dismembratorTM, Braun, Melsungen, Germany). The RNeasyTM Mini Kit (Qiagen, Hilden, Germany) was used for further processing of the samples following the manufacturer's instructions. Tissue powder (approximately 50 mg) was suspended in 300 μl RT-lysis buffer and further homogenized by brief ultrasound sonication (10 s). The homogenates were applied onto QIA shredder columns (Qiagen, Hilden, Germany) and the eluates were subsequently loaded on the extraction columns. Total RNA was eluted with 50 μl DEPC-treated water. To determine RNA concentrations, 5 μl RNA solution was diluted with 75 μl water. Absorption was measured at 260 nm and 280 nm with a photometer (biophotometer, Eppendorf, Hamburg, Germany). Concentrations of the RNA solutions were adjusted to 15 ng RNA/ μl with DEPC-treated water and stored at -70°C until further analysis.

2.1. Reverse transcription

The RT-reaction was carried out with 150 ng total RNA/10 μl . In addition, the reaction mixture contained 4 μl $5\times$ reaction buffer with 250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl_2 , 50 mM dithiothreitol. The concentrations of random primers and deoxy-NTPs were 100 ng and 10 mM, respectively, in a volume of 1 μl each. The mixture was completed with SuperscriptTM RNase H⁻ reverse transcriptase (1 μl containing 200 U) and 1 μl ribonuclease inhibitor (1 U). The final volume was adjusted with DEPC-treated water to 20 μl . All reagents except the ribonuclease inhibitor (Promega, Mannheim, Germany) were purchased from GibcoBRL (Karlsruhe, Germany). Reverse transcription was initiated by an incubation of samples at 22°C for 10 min followed by the enzymatic reaction conducted at 42°C for 50 min. At the end of incubation, the samples were heated at 95°C for 10 min to inactivate the enzyme and denature RNA-cDNA hybrids.

2.2. Real-time PCR

Real-time PCR reactions were based on the 5' nuclease assay (25) which was run on an ABI Prism 7700 Sequence Detection System (TaqManTM, PE Applied Biosystems Foster City, CA, USA). The sequences of primers and probes for the genes analysed in the present study are summarized in Table 1. Primers and probes were chosen with the assistance of the Primer Express Software (PE Applied Biosystems). Oligonucleotides were purchased from Eurogentec (Seraing, Belgium).

Each PCR run included six duplicate cDNA samples of defined concentrations to generate a standard curve (derived from the *in vitro* transcription described below), a no template control and the respective sample cDNAs. Amplification reactions (25 μl) contained 1X TaqManTM universal PCR master mix (PE applied biosystems), 50–900 nM of each primer (for details see

Table 1
Primer sequences and references for IGF1 and AR PCR

| | | | | |
|-------|--|---------------------------------|--------|-------------|
| IGF I | 5'-GTCTGCTTTCACATCTCTTCTACCTG-3' 5'-CCACACACGAAGTGAAGAGCGT-3' | 5'-TTACCAGCTCGGCCACAGCCGGAC-3' | 121 bp | [41] M15481 |
| AR | 5'AGGAAGTGTGATCGCATCATTGC-3' 5'-CTGCCATCATTTCAGGAA-3' | 5'CGCTTCTACCAGCTCACCAAGCTCCT-3' | | [42] M23264 |

Table 1), 175–225 nM probe and 2–4 μ l cDNA. The cycling conditions were 2 min incubation at 50 °C for eliminating carryover PCR products by uracil DNA glycosylase treatment, 10 min at 95 °C for activation of the amplitaq gold DNA polymerase followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

To generate cRNA, each PCR product was cloned into the pCRTM II-TOPO plasmid using the TOPO TA CloningTM Kit following the manufacturer's instructions (Invitrogen, Groningen, The Netherlands). After confirming the sequence and orientation of the cloned PCR product by commercial sequencing (Seqlab, Göttingen, Germany), the plasmid construct was linearized with an appropriate restriction endonuclease and purified using the WizardTM DNA Clean-Up System (Promega, Mannheim, Germany). RNA was synthesized according to the instructions of the manufacturer using the RiboMAXTM Large Scale RNA Production System with either SP6- or T7-polymerase, respectively (Promega, Mannheim, Germany). After photometric determination of concentrations of the RNA stock solutions, serial dilutions (10-fold intervals) of the RNA were reverse transcribed as described above. The standard curve was generated by plotting the known cDNA-concentrations versus the corre-

sponding C_t -value obtained in the real-time PCR reaction using Sequence Detection Software PE Applied Biosystems). To determine the relative expression levels of the tissue samples, the respective- C_t values were interpolated from the standard curve.

2.3. Statistics

Means and standard errors of the means were calculated for prostate weights and 5 α -DHT concentrations. The mRNA of the seminal vesicles were subjected to a quantitative RT-PCR utilizing the real-time TaqMan system. The resulting CT values of the control animals were set 100% and the CT values of the experimental animals calculated in relation to these control values. All data were then subjected to a *T*-test for multiple comparisons. In all figures means and standard errors of the means (S.E.M.) are given. A *P*-value <0.05 was considered statistically significant.

3. Results

Fig. 1 details serum T and DHT levels in the animals fed with T-free or T-containing pellets. Serum T

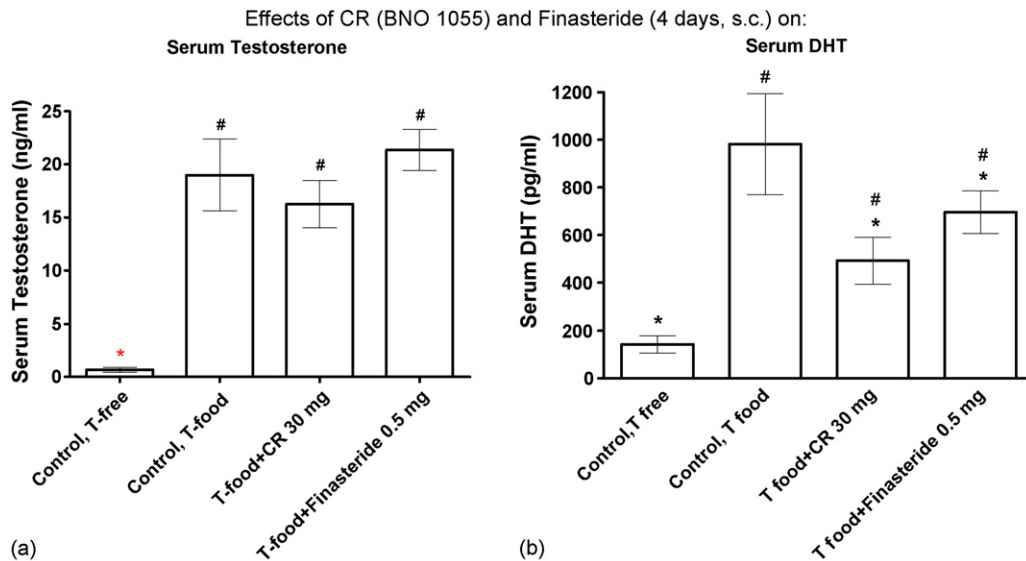


Fig. 1. Serum testosterone (a) levels in all 3 Testosterone treated groups are in a slight supraphysiological range whereas they are almost undetectable in the immature control animals. (b) Serum 5 α dihydrotestosterone (DHT) levels in T-treated animals are significantly elevated above control levels and the *c. racemosa* extract as well as finasteride treatment resulted in lower DHT levels. **p*<0.05 vs. control T-food; #*p*<0.05 vs. T-free food.

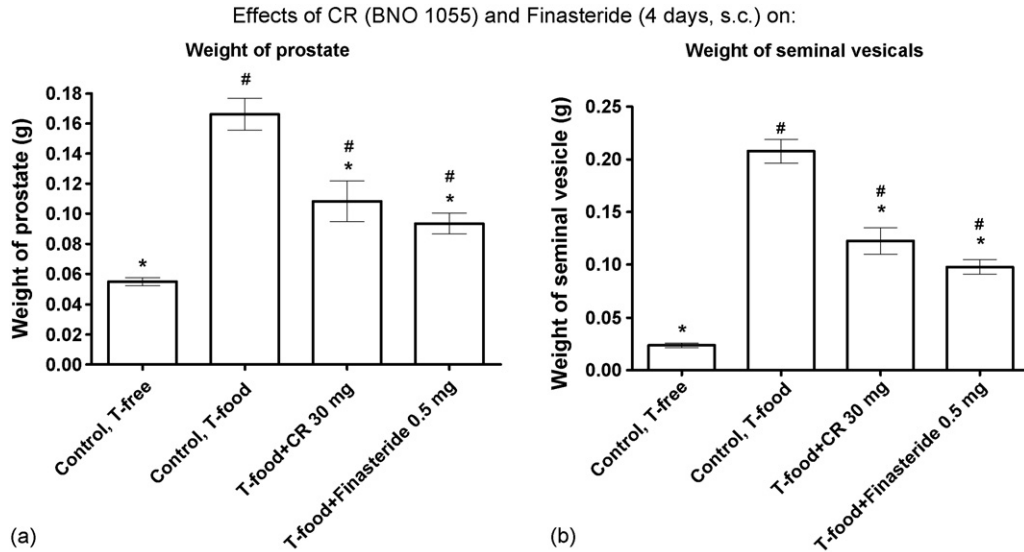


Fig. 2. The testosterone treatment significantly stimulated prostate weight (a) and weight of seminal vesicles (b). Both stimulatory effects of T were significantly reduced by the *c. racemosa* extract and finasteride. * $p < 0.05$ vs. control T-free; # $p < 0.05$ vs. T-free food.

(Fig. 1a) levels were almost undetectable in the controls and in the slightly supraphysiologic range in the T-treated. Co-treatment with CR BNO 1055 or finasteride did not affect serum T-concentrations. Serum DHT in controls were low and significantly higher in the T-

treated animals. This increase was partially prevented by co-treatment with CR BNO 1055 or finasteride (Fig. 1b).

In the T-treated control animals prostate and seminal vesicle weights were almost 4-fold (prostates) and

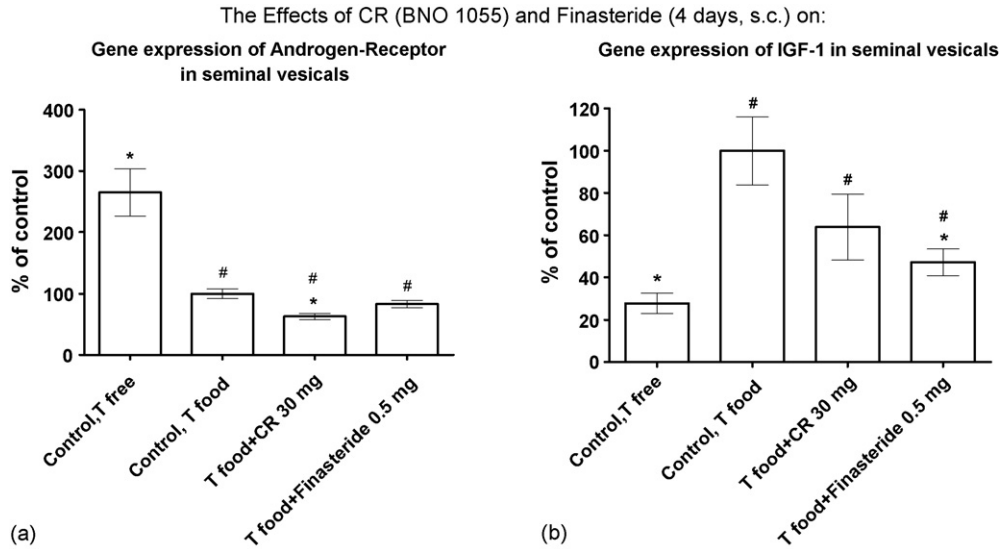


Fig. 3. (a) The testosterone treatment decreased androgen receptor gene expression and a further reduction of the mRNA transcripts were observed under the CR treatment. (b) Gene expression of insulin like growth factor I (IGF-I) was significantly stimulated by T and significantly reduced to intermediate levels by the *c. racemosa* extract and finasteride. * $p < 0.05$ vs. control T-food; # $p < 0.05$ vs. T-free food.

more than 5-fold (seminal vesicles) increased in comparison to the animals not treated with the androgen (Fig. 2). The T-induced increased weights of prostates and seminal vesicles were significantly reduced by the 30 mg CR BNO 1055 extract and this effect was equipotent to the 0.5 mg of finasteride (Fig. 2a and b).

Gene expression of the androgen receptor and of insulin like growth factor 1 (IGF1) in the seminal vesicles was downregulated in the T-treated animals and further decreased in the CR BNO 1055 fed animals (Fig. 3a). Expression of the IGF I gene was upregulated by T and both, finasteride and CR decreased gene expression of this growth factor (Fig. 3b).

4. Discussion

In earlier cell biological studies it was shown that inhibition of 5 α -reductase inhibits growth of prostate cancer-derived cell lines [7,8]. In the recently published prostate cancer prevention trial the administration of the synthetic 5 α -reductase inhibitor finasteride yielded an almost 25% reduction of prostate cancers following 7 treatment years [13]. Even though the malignancy as determined by Gleason grading was higher in the finasteride when compared to the placebo treated men [13], this marked reduction in prostate cancer occurrence is promising and invites for further studies with other compounds. As a first step other compounds with 5 α -reductase inhibiting properties need to be identified. A simple means to test such effects in vivo is measurement of T-induced prostate proliferation as determined by the weights of the organ. We observed in intact male rats a reduction of prostate weights under the treatment with the *C. racemosa* extract BNO 1055 (unpublished). This prompted us to study whether CR BNO 1055 contains substances with 5 α -reductase inhibitory properties. To test this working hypothesis we utilized a modified Hershberger assay using instead of orx immature rats the 24-day-old intact rats and compared the effects of the extract with those of finasteride. Finasteride is known to inhibit testosterone-induced growth of various prostate cell lines and of prostates in orx immature rats due to inhibition of the conversion of testosterone to 5 α -DHT, the main androgen in the prostate [2,8]. We modified this model by using 24-day-old intact male rats in which the prostates had never developed and indeed, the oral application of testos-

terone with the food resulted in a 3- or 5-fold increase of prostate and seminal vesicle weights, respectively. Serum T-concentrations induced by the T-treatment resulted in slightly supraphysiologic values. A small fraction of the circulating T was converted to DHT such that the serum DHT concentrations were readily measurable. In agreement with earlier published results [31] this treatment with finasteride does not alter serum T-concentrations significantly as only small amounts of T are converted to DHT. Treatment with finasteride the inhibitor of 5 α -reductase I and II however reduced serum DHT concentrations indicating the effectiveness of this enzyme inhibitor. An even more marked reduction of serum 5 α -DHT was achieved by the treatment with the CR BNO 1055 extract. Hence, this extract contains 5 α -reductase inhibitors. It is therefore not surprising that not only finasteride but also CR BNO 1055 reduced the testosterone-induced growth of both glands. A similar weight reduction was also found in the seminal vesicles of CR BNO 1055-treated animals. The stimulatory actions of 5 α -DHT in the prostate are mediated androgen receptor (AR) and one part in the chain of events leading to prostate growth is stimulation of insulin like growth factor 1 and the same mechanisms appear to take place in the seminal vesicles which are also stimulated by 5 α -reduced androgens [32,33]. The seminal vesicles of the animals were used to determine mRNA transcript concentrations of AR and IGF1. As T stimulated IGF I gene expression and this could be effectively inhibited by finasteride and the CR extract BNO 1055 confirming again the similarity of action of this extract with finasteride. It is therefore likely that the protein production of the proliferation-promoting factor IGF1 is also significantly reduced by both treatments thereby reducing the proliferation of prostate cells. Published data about suppressive effects of androgens on AR receptor gene expression in the accessory sex organs are in agreement with the present data [28,34]. The further reduction of AR gene expression by CR BNO 1055 may be beneficial for the prostate as this may reduce that the sensitivity of the accessory sex organs to androgens. These results make it again likely that compounds in the extracts of black cohosh contain a 5 α -reductase inhibitor. The chemical configuration of this or these 5 α -reductase inhibitor(s) is not yet known but for the following reasons it can be assumed that the compound(s) is/are at least as potent as finas-

teride: the treatment with 0.5 mg finasteride per animal was equipotent to the treatment with 30 mg CR BNO 1055. Although the active 5α -reductase inhibitory substances are unknown at present it is highly unlikely that they contribute with more than 1% to the constituents of CR BNO 1055, i.e. less than 0.3 mg are likely to be the 5α -reductase inhibitory principles. Therefore, the conclusion that the CR BNO 1055 constituent(s) is/are more active than finasteride appears to be justified.

Estrogens play also a role in regulating prostate growth: stimulation of proliferation of the human prostate cancer-derived cell LNCaP was demonstrated under estrogens [16] and this was inhibited by the antiestrogen ICI 182780 [35]. Hence, the effects appear to be mediated by estrogen receptors. It is currently controversially discussed whether extracts of black cohosh contain estrogenic compounds: Liu et al. [36] found no estrogenic effects, Zierau et al. [37] demonstrated antiestrogenic activities and the same group of authors showed later coactivation of cells via the ER β [38]. Hence, the situation as to whether CR extracts may act via estrogen receptors is confusing. We showed recently that CR BNO 1055 inhibits arylhydrocarbon receptor (AhR) mediated effects [16]. Since the AhR nuclear translocator ARNT is a coactivator of both estrogen receptors [39] it is possible that not yet identified compounds in CR BNO 1055 act via the AhR/ARNT pathway thereby indirectly modulating ER activity. AhR selective modulators are also discussed as putative anticarcinogenic compounds [40].

In preliminary unpublished experiments we showed that BNO 1055 inhibits also 5α -reductase I in a skin fibroblast-derived cell line. Hence, it is possible that the CR extract BNO 1055 may also be effective to inhibit the function of sebaceous glands and thereby acne and possibly also hirsutism and androgenetic alopecia.

In summary, we have shown that the CR extract BNO 1055 contains one or more potent 5α -reductase inhibitor(s) thereby preventing the conversion of testosterone to 5α -dihydro-testosterone which is the bioactive androgen in the prostate and in the seminal vesicles. These potent 5α -reductase inhibitor(s) may prove to be useful in aging men to prevent benign prostate hyperplasia as well as the development of prostate cancer.

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