Disruption of Androgen and Estrogen Receptor Activity in Prostate Cancer by a Novel Dietary Diterpene Carnosol: Implications for Chemoprevention

Jeremy J. Johnson1,3, Deeba N. Syed2, Yewseok Suh2, Chenelle R. Heren1, Mohammad Saleem2, Imtiaz A. Siddiqui2, and Hasan Mukhtar2

Abstract

Emerging data are suggesting that estrogens, in addition to androgens, may also be contributing to the development of prostate cancer (PCa). In view of this notion, agents that target estrogens, in addition to androgens, may be a novel approach for PCa chemoprevention and treatment. Thus, the identification and development of nontoxic dietary agents capable of disrupting androgen receptor (AR) in addition to estrogen receptor (ER) could be extremely useful in the management of PCa. Through molecular modeling, we found that carnosol, a dietary diterpene, fits within the ligand-binding domain of both AR and ER-α. Using a time-resolved fluorescence resonance energy transfer assay, we found that carnosol interacts with both AR and ER-α and additional experiments confirmed that it functions as a receptor antagonist with no agonist effects. LNCaP, 22Rv1, and MCF7 cells treated with carnosol (20-40 μmol/L) showed decreased protein expression of AR and ER-α. Oral administration of carnosol at 30 mg/kg 5 days weekly for 28 days to 22Rv1 PCa xenografted mice suppressed tumor growth by 36% (P = 0.028) and was associated with a decrease in serum prostate-specific antigen by 26% (P = 0.0042). These properties make carnosol unique to any known antiandrogen or antiestrogen investigated thus far for the simultaneous disruption of AR and ER-α. We suggest that carnosol may be developed or chemically modified through more rigorous structure-activity relationship studies for a new class of investigational agents—a dual AR/ER modulator. Cancer Prev Res; 3(9); 1112–23. ©2010 AACR.

Introduction

Evidence is emerging that androgens may not be the only hormone responsible for the pathogenesis of prostate cancer (PCa); recent data have suggested that estrogens may be another important consideration in the PCa puzzle (1–3). Using Noble rats, the combined treatment of testosterone and estradiol (E2), but not the separate administration of each, has been shown to significantly induce dysplasia and increase the mitotic index in the dorsolateral prostate (4). Another animal model has evaluated the de novo synthesis of E2 from testosterone via the aromatase enzyme by generating ARKO (aromatase knock-out) mice. Three characteristics were associated with these mice that included (a) increased serum levels of androgens, (b) complete absence of serum E2, and (c) they were incapable of developing PCa (5). More recently, estrogen receptor-α (ER-α) has been receiving increased attention in PCa as evidenced by clinical trials using estrogen antagonists as a monotherapy or in combination with androgen antagonists with encouraging results (2). Further support to the concept of estrogen as a target in PCa comes from a clinical trial in participants (n = 447) randomized to the antiestrogen toremifene (20 mg/d) as a monotherapy for 1 year, which showed a decreased cumulative risk of progressing from high-grade prostatic intraepithelial neoplasia to PCa by 21.8% (P < 0.05; ref. 6).

Simultaneous disruption of both androgen receptor (AR) and ER has been proposed with Food and Drug Administration (FDA)–approved drugs such as toremifene and fulvestrant as well as other agents; however, there are significant limitations with these chemical entities. Toremifene has a bimodal effect where it functioned as an antagonist at lower concentrations; however, as the dose was increased, it functioned as an agonist in LNCaP cells (7). Limitations of fulvestrant as a dual AR and ER modulator include (a) the AR antagonist effect is saturable regardless of increasing the dose, (b) it cannot bind to the mutated AR (T877) that is found in LNCaP cells, and (c) it did not exhibit any effect...
in a phase II clinical trial in castration-resistant PCa (8, 9). Aromatase inhibitors such as exemestane have also been proposed as a way to target both estrogen and androgen signaling; however, they also function as AR agonists (10, 11).

Carnosol (Fig. 1A) is a dietary diterpene isolated from culinary herbs that include rosemary, basil, sage, and oregano and has been noted for its potent antioxidant activity and anticancer properties. Approximately 5% of the dry weight of rosemary leaves are the diterpenes carnosol and carnosic acid (12), being responsible for 90% of the antioxidant activity found in rosemary (13, 14). In traditional Chinese medicine, rosemary extracts containing high amounts of diterpenes and triterpenes are used to treat inflammatory conditions such as arthritis. Additionally, dietary supplements of rosemary extracts standardized to carnosol and/or carnosic acid are available in health food markets. An in vivo study that evaluated the antimutagenic activity of rosemary and carnosol was associated with a significant decrease, 74% and 65%, respectively, in the number of 7,12-dimethylbenz(a)anthracene–induced mammary adenocarcinomas when compared with controls (15). Another in vivo study showed that dietary carnosol (0.1%) decreased adenomatous polyposis coli (APC) associated adenoma formation by 46% in the C57BL/6j/Min/+ (Min/+) mouse compared with controls (16). Recently, we have shown that carnosol induces cell cycle arrest by targeting AMP-activated protein kinase, leading to an inhibition of the mammalian target of rapamycin pathway (17).

Here, we provide evidence that carnosol, a dietary diterpene, functions as a dual AR and ERα modulator by binding...
and disrupting AR and ER-α receptor, leading to a decrease in protein stability of AR and ER-α. Carnosol along with other diterpenes (18) may perhaps prove useful by themselves or as a lead compound for the development of a dual AR/ER-α modulator for use in the chemoprevention and/or chemotherapy of hormone-responsive cancers.

**Materials and Methods**

The AR and prostate-specific antigen (PSA) antibodies were obtained from Santa Cruz Biotechnology, Inc., and ER-α antibodies were obtained from Cell Signaling Technology, Inc. Carnosol (>99% pure) was purchased from Cayman Chemical. Tamoxifen (≥99%) and flutamide (≥99%) were purchased from Sigma. Anti-mouse and anti-rabbit secondary antibody horseradish peroxidase conjugate was obtained from Cell Signaling Technology. Protein assay kit was obtained from Pierce. PSA ELISA kit was purchased from Anogen.

**Cell culture and treatment**

The LNCaP, 22Rv1, and MCF7 cells were obtained from the American Type Culture Collection. These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Human normal prostate epithelial cells (PrEC) were obtained from Lonza and grown according to the manufacturer’s instructions. All cells were maintained under standard cell culture conditions at 37°C and 5% CO2 environment. Carnosol dissolved in DMSO was used for the treatment of cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. The cells (60-70% confluent) were treated with carnosol (10-60 μmol/L) for 24 to 48 hours in complete growth medium.

**Cell viability**

Cell viability of human cells treated with carnosol was determined by MTT assay as described previously (17).

**Molecular modeling**

Molecular modeling of carnosol was done with the appropriate nuclear receptor from a protein database bank and with AutoDock 4.2 by the Small Molecule Screening Facility at the University of Wisconsin-Madison.

**Biochemical nuclear receptor activity assay**

To identify AR and ER-α ligands, a fluorescence resonance energy transfer (FRET)–based assay was used to test the antiantidrogenic and antiestrogenic potential of carnosol using purified protein of AR and ER-α that contain the ligand-binding domains (LBD). R1881 was included as the control competitor. Briefly, in this assay, the ligands for AR or ER-α are identified by their ability to compete with and displace a strong-affinity AR or ER-α ligand (called fluoromone) from the receptor. A purified, glutathione S-transferase (GST)–tagged AR-LBD or ER-α-LBD (1 nmol/L) is indirectly labeled with a terbium-labeled anti-GST tag antibody (5 nmol/L). Binding of fluoromone (1 μmol/L solution) to AR or ER-α is then measured by monitoring FRET from the terbium-labeled anti-GST tag antibody to the red fluorescent ligand, resulting in a high time-resolved FRET (TR-FRET) ratio (570-nm fluorescent emission/546-nm fluorescent emission). Competitors will displace Fluormone AL Red and disrupt FRET, resulting in a lower TR-FRET ratio. Serial dilutions of R1881 and carnosol were done in 100% DMSO at 100× the final screening concentration. Each dilution series was then diluted to 4× the final screening concentration in assay buffer (Invitrogen). To do the assay, 5 μL of 4× carnosol and R1881 were dispensed in the 384-well assay plate. Following this, 5 μL of 4 nmol/L AR or ER-α LBD were dispensed in the assay wells followed by the addition of 10 μL of 10 nmol/L terbium anti-GST antibody/20 nmol/L fluoromone mixture. The plates were incubated for 2 hours at room temperature and read using 546- and 570-nm emission intensities on Tecan Infinite F500 plate reader, following excitation at 340 nm. A 100-μs delay followed by a 200-μs integration time was used to collect the time-resolved signal. Three-fold recurrently increasing concentrations of reference competitor R1881 (starting from 0.001 to 10,000 nmol/L, 10 different concentrations) and carnosol (starting from 0.001 to 100,000 nmol/L) were used to test for competition with a constant 10 nmol/L concentration of the labeled competitor fluoromone. The concentration of tracer corresponds to its KD value for binding to our AR-LBD. Compound interference is defined in these cases as >50% quenching of the terbium reference emission intensity measured at 546 nm. No interference with carnosol was observed during the assay.

**Cell-based nuclear receptor reporter assays**

AR-UAS-bla GripTite 293 cells contain the LBD of the human AR fused to the DNA-binding domain (DBD) of GAL4 stably integrated in the GeneBLAzer UAS-bla GripTite 293 cell line. ER-α-UAS-bla GripTite 293 cells contain the LBD of ER-α fused to the DBD GAL4 stably integrated in the GeneBLAzer UAS-bla GripTite 293 cell line. GeneBLAzer UAS-bla GripTite cells stably express a β-lactamase (bla) reporter gene under the transcriptional control of an upstream activator sequence (UAS). When an agonist binds to the LBD of the GAL4 (DBD)–AR (LBD) fusion protein, the protein binds to the UAS, resulting in expression of bla. With expression of bla, the substrate is cleaved, leading to a separation of the fluorophores, disrupting the energy transfer. The manufacturer’s protocol was followed for screening of carnosol in agonist and antagonist modes.

**Agonist screen.** Four microliters of a 1× serial dilution of R1881 or E2 and carnosol were added to appropriate wells of a 384-well tissue culture treated assay plate. Cell suspension (32 μL, 10,000 cells) was added to each well. Assay medium (4 μL) was added to all wells to bring the final assay volume to 40 μL. The plate was incubated for 16 hours at 37°C/5% CO2 in a humidified incubator. Eight microliters of 1 μmol/L substrate loading solution were added to each well, and the plate was incubated for 2 hours at room temperature. The plate was read on a fluorescence plate reader with two scans done at 409/460 and 409/530 nm.
Antagonist screen. Four microliters of a 10× serial dilution of 4-hydroxytamoxifen or cyproterone acetate and carnosol were added to appropriate wells of a 384-well TC-treated assay plate. Cells (32 μL) were added to the wells and preincubated at 37°C/5% CO₂ in a humidified incubator with compounds and control antagonist for 30 to 60 minutes. Four microliters of 10× control agonist R1881 at the predetermined EC₅₀ concentration were added to wells containing the control antagonist or compounds. The plate was incubated for 16 hours at 37°C/5% CO₂ in a humidified incubator. Eight microliters of 1 μmol/L substrate loading solution were added to each well, and the plate was incubated for 2 hours at room temperature and read on a fluorescence plate reader with two scans done at 409/460 and 409/530 nm.

Western blot
Following treatment of cells by carnosol, whole-cell lysates were collected and used to do Western blot analysis as described previously (17). Cells were lysed and centrifuged at 14,000 × g for 25 minutes, and the supernatant fraction was used for immunoblotting. Proteins were resolved on a 12% Tris glycine gel and transferred onto a nitrocellulose membrane. After blocking with 5% nonfat dry milk in blocking solution, the membrane was incubated with the desired primary antibody for 2 hours at room temperature. The membrane was then incubated with the appropriate peroxidase-conjugated secondary antibody, and the immunoreactive bands were visualized using the SuperSignal West Pico chemiluminescent substrate (Pierce) kit. To ensure equal protein loading, each membrane was stripped and reprobed with β-actin antibody to normalize for differences in protein loading.

AR and ER-α protein stability assay
LNCaP cells were treated with 40 μmol/L carnosol and 50 μg/mL cycloheximide for 0, 6, 12, and 24 hours, followed by the preparation of whole-cell lysates. Cycloheximide was added to the medium 30 minutes before the addition of carnosol. AR and ER-α protein levels were determined by Western blot analysis with antibody specifically against AR and ER-α and normalized to β-actin control.

Immunofluorescence microscopy
Cells were seeded on a two-chamber tissue culture–treated glass slides as described previously (19). The following day, medium was replaced with or without carnosol and cultured for 24 hours. After removing the chamber, slides were rinsed with PBS and cells were fixed with 2% paraformaldehyde and permeabilized in methanol. After washing with PBS, slides were blocked with 2% donkey serum. Primary and secondary antibodies were incubated in 5% donkey serum. SlowFadeGold-DAPI (Invitrogen) was used as mounting and counterstaining medium. For analysis, Bio-Rad Radiance 2100 MP Rainbow system in the W.M. Keck Laboratory for Biological Imaging at the University of Wisconsin-Madison was used.

Transient transfection and luciferase assays
Transient transfections were done using Lipofectamine (Invitrogen) per the manufacturer’s protocol using an AR luc (~6-kb promoter) in the pGL3-Basic vector and an ER-α luc (~5.8-kb) in the pGL2-Basic vector reporter plasmids provided by Donald J. Tindall and Ronald J. Weigel, respectively. Cotransfections were done with 0.5 μg of reporter plasmid and 15 ng of Renilla luciferase plasmid (Promega), and assays were done after 24 hours using dual-luciferase assay kit (Promega). Cells were cotransfected and normalized with Renilla luciferase plasmid (Promega). Following transfection, cells were treated with carnosol for 24 hours and samples were prepared per the manufacturer’s protocol.

PSA ELISA
The human PSA ELISA kit (Anogen) was used for the quantitative determination of PSA levels in the mouse serum per the manufacturer’s protocol. Following collection of blood, the samples were allowed to sit at room temperature for 1 hour and the sera were separated by centrifuging at 2,000 rpm for 15 minutes at 4°C and then stored at −20°C until assayed for secreted PSA. A standard curve was plotted using available PSA standards (2, 10, 20, 40, and 80 ng/mL) to quantify individual samples.

Cleaved (active) caspase-3 ELISA
Lysates were prepared with the same lysis buffer for Western blotting. Two hundred micrograms of lysates were analyzed using PathScan cleaved caspase-3 sandwich ELISA (Cell Signaling Technology) following the manufacturer’s instructions. Lysates were mixed with 100 μL of sample diluent and incubated in antibody-coated microwell strips. One hundred microliters of cleaved caspase-3 detection antibodies were added to each well. Binding was detected with 100 μL of horseradish peroxidase–linked streptavidin antibody and 100 μL of 3,3′,5,5′-tetramethylbenzidine substrate solution. The colored reaction product was measured in a standard ELISA plate reader at 450 nm.

In vivo 22Rv1 tumor xenograft model
Athymic (nu/nu) male nude mice (7 weeks old; Harlan Laboratory) were housed under pathogen-free conditions with a 12-hour light/12-hour dark schedule and fed with an autoclaved diet ad libitum. AR- and ER-α–positive 22Rv1 cells were used for determining the in vivo effects of carnosol because these cells form rapid and reproducible tumors in nude mice and secrete significant amounts of PSA in the bloodstream of the host. To establish tumor xenografts, mice were injected s.c. with 1 × 10⁶ 22Rv1 cells mixed with 50 μL RPMI 1640 + 50 μL Matrigel (Becton Dickinson) in the right and left flanks of each mouse. Fourteen animals were then randomly divided into two groups, with seven animals in group 1 and seven animals in group 2. The animals in group 1 received cottonseed oil (100 μL) by oral gavage and served as control. The animals in group 2 received carnosol (30 mg/kg) in 100 μL of cottonseed oil by oral gavage 5 days weekly. Body weights...
were recorded 5 days weekly throughout the study. Once xenografts started growing, their sizes were measured thrice weekly. The tumor volume was calculated by the formula $0.5238L_1L_2H$, where $L_1$ is the long diameter, $L_2$ is the short diameter, and $H$ is the height of the tumor. This formula is derived from an equation for calculating the volume of a hemi-ellipsoid, the geometric figure most nearly approximating the shape of tumors (20). All animals in groups 1 and 2 were sacrificed when tumors reached an average tumor volume of $\sim 1,200$ mm$^3$ in the control group. All procedures conducted were in accordance with the guidelines for the use and care of laboratory animals. Blood samples were collected by the “mandibular bleed.” The blood was allowed to sit at room temperature for 1 hour, and the sera were separated by centrifuging blood at 2,000 rpm for 15 minutes at 4°C and then stored at $-20$°C for future assays.

Statistical analysis
All statistical analysis was done by using GraphPad QuickCals software. Statistical significance of differences in all measurements between control and treated groups was determined. Student’s paired $t$ test was used for pairwise group comparisons for analyzing luciferase assays, tumor measurements, and serum PSA levels. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Carnosol decreases cell viability of LNCaP, 22Rv1, and MCF7 cells in a dose-dependent manner
Given the structural similarity of carnosol to dihydrotestosterone (DHT) and $E_2$ (Fig. 1A), we hypothesized that carnosol may have the ability to interact with AR and ER-$\alpha$. Molecular modeling showed that carnosol is able to fit within the LBD of both AR and ER-$\alpha$ (Fig. 1B). Based on this premise, three different cancer cell lines were selected that express both AR and ER-$\alpha$ that included PCA cell lines LNCaP and 22Rv1 and the breast cancer cell line MCF7 (Fig. 1C). Carnosol decreased cell viability in a dose-dependent manner, with an IC$_{50}$ of 19.6 µmol/L in LNCaP cells, 22.9 µmol/L in 22Rv1 cells, and 25.6 µmol/L in MCF7 cells (Fig. 1C). This decrease in cell viability was accompanied by a change in cellular morphology in carnosol-treated cells (Fig. 1D).

Carnosol binds to AR and ER-$\alpha$ in a cell-free biochemical-based assay
The role of AR and ER-$\alpha$ in PCa is well established, and subsequently, androgen depletion pharmacotherapy and estrogen depletion pharmacotherapy are emerging as targets of interest in both prostate and breast cancer. We evaluated carnosol for its interaction with AR and ER-$\alpha$ using a LanthaScreen TR-FRET (i.e., cell-free biochemical-based assay) competitive binding assay. Using this assay, we found that carnosol bound to AR with an IC$_{50}$ of 40.8 µmol/L and to ER-$\alpha$ with an IC$_{50}$ of 51 µmol/L as shown in Fig. 2A and B, respectively. These data provided evidence that carnosol can bind to AR and ER-$\alpha$; however, further evaluation was needed to determine if carnosol displays agonist/antagonist properties.

Carnosol does not display agonist activity at the AR or ER-$\alpha$ in a cell-based assay
To determine if carnosol possesses agonist activity, we used the AR-UAS-bla GripTite 293 cells and the ER-$\alpha$-UAS-bla GripTite 293 cells that have a LBD for the appropriate nuclear receptor. Carnosol was not found to display any agonist activity in either the AR assay (Fig. 2C) or the ER-$\alpha$ assay (Fig. 2D) at doses up to 100 µmol/L. These results suggest that carnosol altogether physically interacts with AR and ER-$\alpha$ as shown by the cell-free biochemical-based assay, and it possesses no agonist activity.

Carnosol displays antagonist activity at the AR and ER-$\alpha$ in a cell-based assay
To determine if carnosol has antagonist activity, we used the AR-UAS-bla GripTite 293 cells as described above with a slight modification, where cells were stimulated with the known agonist R1881 (AR) or $E_2$ (ER-$\alpha$) and treated with carnosol. A dose-response effect with regard to antagonist activity was observed with carnosol, with an estimated EC$_{50}$ of 29.6 µmol/L for AR (Fig. 2C) and an EC$_{50}$ of 32.9 µmol/L for ER-$\alpha$ (Fig. 2D). The results of this further support that carnosol does not display agonist activity at doses up to 100 µmol/L and functions as an antagonist for both AR and ER-$\alpha$.

Carnosol decreases the expression of AR, ER-$\alpha$, and PSA in LNCaP, 22Rv1, and MCF7 cells
As described above, we used cells stimulated with a known agonist (R1881 or $E_2$) and found that carnosol has antagonist properties and no agonist properties. Next, we evaluated three different cell lines to determine if carnosol can decrease the functionality (i.e., protein expression) of AR and ER-$\alpha$. First, whole-cell lysates were evaluated for the intracellular expression of AR, ER-$\alpha$, and PSA after a 48-hour carnosol treatment (20, 40, and 60 µmol/L) using PCA cell lines (LNCaP and 22Rv1) and a breast cancer cell line (MCF7). Our results show that after 48 hours, carnosol decreased the protein expression of AR and ER-$\alpha$ in a dose-dependent manner in LNCaP and 22Rv1 prostate cell lines and MCF7 breast cancer cell line (Fig. 3A). In addition, we evaluated the time-dependent (0, 6, 12, and 24 hours) decrease in AR and ER-$\alpha$ in LNCaP cells and observed a decrease in protein expression as early as 6 hours after treatment (Fig. 3B).

Carnosol alone or in combination with cycloheximide decreases the expression of AR and PSA in a time-dependent manner
To further understand the effect of carnosol on AR stability, we did a pulse-chase experiment using the translation inhibitor cycloheximide. LNCaP cells treated with either carnosol or cycloheximide led to a time-dependent

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decrease in AR and ER-α protein expression as expected (Fig. 3B). Interestingly, cotreatment of LNCaP cells with cycloheximide and carnosol with LNCaP cells further increased the rate of AR and ER-α degradation, suggesting that carnosol destabilizes AR and ER-α protein levels.

Carnosol decreases the mRNA expression of AR and ER-α
To determine if carnosol acts directly on the AR and ER-α promoter to inhibit gene transcription, we examined the effect of carnosol on the activity of an AR and ER-α promoter-reporter construct transfected into LNCaP cells. LNCaP cells were transiently transfected with promoter-reporter constructs for AR (∼6 kb) and ER-α (∼5.8 kb). A Renilla luciferase plasmid was used as a control for transfection efficiency. As shown in Fig. 4A and B, carnosol treatment significantly (P < 0.05) decreased both AR and ER-α promoter-luciferase activity compared with vehicle treatment.

Carnosol decreases the protein expression of AR and ER-α in LNCaP cells
To further confirm whether carnosol can decrease the expression of AR and ER-α, LNCaP cells were grown in chamber slides for 24 hours and treated with carnosol for 24 hours and prepared as described in Materials and Methods. We observed a decrease in overall protein expression of both receptors when LNCaP cells were treated with carnosol (Fig. 4C and D). In addition, the translocation from the cytoplasm to the nucleus of AR and ER-α was inhibited by carnosol, as shown by a diminished fluorescence in the AR and ER-α panels along with the merged panel containing the cells counterstained with 4′,6-diamidino-2-phenylindole (DAPI).
Carnosol displays limited effects on PrECs

To determine if carnosol decreases cellular viability of nontumorigenic cells, PrECs were treated with increasing concentrations of carnosol, flutamide, or tamoxifen for 48 hours and remained intact as visualized in Fig. 5A. At the highest dose of 60 μmol/L, the decrease in cell viability was 76.0 ± 2.6% (carnosol), 60.3 ± 3.2% (flutamide), and 68.4 ± 5.6% (tamoxifen) as shown in Fig. 5B. These results were unique compared with tumorigenic cell lines as shown in Fig. 1. Whole-cell lysates were prepared and used to detect cleaved (activated) caspase-3 of carnosol-treated 22Rv1 cells. Carnosol treatment for 24 hours led to a significant increase \((P < 0.01)\) in cleaved caspase-3 versus control as shown in Fig. 5C. These results suggest that carnosol is as tolerable to nontumorigenic cell lines (e.g., PrEC) as flutamide and tamoxifen that are used regularly in clinical settings.

Carnosol reverses the upregulation of AR and ER-α induced by flutamide or tamoxifen

LNCaP cells were treated with carnosol, flutamide, tamoxifen, carnosol/flutamide, carnosol/tamoxifen, or tamoxifen/flutamide for 24 hours. In agreement with other investigators (7, 21–23), we observed an increase in AR and ER-α activity with treatment of flutamide or tamoxifen individually, as shown by Western blot analysis in Fig. 5C. However, on cotreatment of flutamide or tamoxifen with carnosol, a decrease in protein expression of AR and ER-α was observed. We also observed a similar decrease in protein expression of AR and ER-α when cotreatment of tamoxifen and flutamide was done.

Carnosol inhibits the growth of human prostate carcinoma 22Rv1 cells and PSA secretion in athymic nude mice

Athymic nude mice were implanted with AR- and ER-α–positive 22Rv1 cells and divided into two cohorts receiving either cottonseed oil (i.e., vehicle) or carnosol (30 mg/kg). Carnosol administered orally 5 days weekly seemed to be tolerable as depicted by daily body weight measurements (Fig. 6A). On day 12, the appearance of small tumors was observed in both cohorts, with consistently a smaller average tumor volume being observed in the mice treated with carnosol as depicted in Fig. 6B. The average computed tumor volume was significantly inhibited in mice receiving carnosol. In the control group, the average tumor volume of 1,257 mm³ was reached at day 28 after tumor cell inoculation, whereas mice receiving carnosol had an average tumor volume of 813 mm³, representing a significant suppression in tumor growth by 36% \((P = 0.028)\). In addition, protein lysates were evaluated from carnosol-treated mice and analyzed by Western blot analysis. Overall, we observed a decrease in AR and ER-α protein expression in mice treated with carnosol as shown in Fig. 6C.

At the conclusion of the study on day 28, blood was collected through mandibular bleed. A quantitative sandwich ELISA was used to determine total circulating PSA in mouse...
serum secreted by the implanted 22Rv1 tumor cells. Significant inhibition of PSA secretion was observed in carnosol-treated mice as shown in Fig. 5D. At day 28, PSA levels were 15.2 ng/mL in the control cohort and 11.2 ng/mL in the carnosol-treated cohort. Taken together, we show that the treatment of mice with carnosol when given orally inhibited tumor formation by 36% (P = 0.028) and suppressed serum levels of PSA by 26% (P = 0.0042).

Fig. 4. A and B, LNCaP cells were transiently transfected with an ∼6-kb AR promoter-luciferase reporter plasmid in a pGL3-Basic vector or an ∼5.8-kb ER-α promoter-luciferase reporter plasmid in a pGL2-Basic vector. Eighteen hours after transfection, cells were treated with DMSO (vehicle) or 30 μmol/L carnosol and analyzed for luciferase activity after 24-h treatment with carnosol. Columns, mean of three individual samples; bars, SD. *, P < 0.01. C and D, LNCaP cells were grown in chamber slides at 100,000 per chamber overnight and treated with carnosol (30 μmol/L) for 24 h. For labeling, anti-AR antibody (Santa Cruz Biotechnology) and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) were used as primary and secondary antibodies after 24-h carnosol treatment, respectively. For labeling, anti-ER-α (Cell Signaling Technology) and Alexa Fluor 594 goat anti-mouse IgG were used after 24-h treatment. DAPI was used to counterstain the nucleus.
Discussion

For a long time, the role of androgens in PCa has been associated with androgens stimulating the AR. Recently, it has become increasingly clear that there are two significant shortcomings to the strategy of inhibiting a single isolated target for PCa. First, the role of antiandrogens is limited because androgen antagonists (e.g., flutamide and bicalutamide) are used clinically for only 6 to 12 months to control the initial "flare" of PCa. This is often followed by an eventual conversion of PCa to androgen-independent status with studies suggesting an upregulation of AKT (24) and AR (25). Second, a host of adverse events associated with androgen antagonists can be observed that include hyperlipidemia (26), osteoporosis (26, 27), and gynecomastia (28). More recently, a role for estrogens stimulating ER-α in the prostate gland is being more closely scrutinized in the etiology of PCa. Specifically, preclinical models have begun to shed light on the role of androgens and estrogens in etiology of PCa. This has been observed in Noble rats that require the coadministration of both testosterone and E2 for the initiation of PCa (4). Furthermore, animal studies have shown that the aromatase enzyme responsible for the conversion of testosterone to E2 is essential for the development of PCa (5). In human clinical settings, considerable evidence has emerged that supports an association with estrogen and PCa. It is interesting that the relative levels of E2 increase with age, as does the risk of PCa, and may contribute to PCa risk (29). Epidemiologic studies have suggested that African Americans, who are at the highest risk of PCa, display the highest levels of estrogens (30). Further support of the role for estrogens in PCa initiation and progression comes from a study where participants were diagnosed with high-grade prostatic intraepithelial neoplasia, a risk factor for PCa, and when treated with an antiestrogen
toremifene showed a decreased cumulative risk of PCa diagnosis by 21.8% (6).

As a strategy to decrease unwanted effects of hormone deprivation therapy, clinical trials have combined antiandrogens and antiestrogens to improve lipid profile (26), increase bone mineral density (27, 31), and reduce gynecomastia (28, 32) in human subjects. These side effects, along with other considerations such as androgen resistance, represent a significant limitation and are often the explanation for their limited clinical use of 6 to 12 months. By incorporating both an antiestrogen and an antiandrogen, this strategy may allow for the decrease of known side effects of antiandrogens, a reduction in resistance to antiandrogens due to the multitarget strategy, and possibly the potential to introduce regimen earlier in the disease development as a chemopreventive agent. In addition, by limiting the adverse events, it may not be unreasonable to hypothesize that targeting AR and ER-α will allow for the therapy to be used earlier and for a longer period of time.

Here, for the first time, we report that carnosol, a novel dietary diterpene, inhibits the growth of androgen-dependent PCa cells. More importantly, the possible role of carnosol to be developed as a chemopreventive agent is strengthened by our observation that carnosol displays minimal effect on the growth of normal PrECs (Fig. 5A and B). In this context, when HEK293 cells were treated with carnosol up to 1 mmol/L, there was not a noticeable decrease in cell viability or change in cellular morphology (data not shown). Carnosol was found to physically interact with the LBD of both AR and ER-α. In cells stimulated with R1881 or E2, carnosol was shown to display purely antagonist properties with no evidence of agonist properties at the AR and ER-α. This is a unique property compared with tamoxifen, toremifene, and fulvestrant that have been
evaluated for the dual disruption of AR and ER-α. When administered orally to athymic nude mice with 22Rv1 xenografts, carnosol inhibited tumor formation by 36%.

It is possible that carnosol on binding to the LBD of AR and ER-α could result in the destabilization of AR and ER-α, thereby decreasing its expression. Support for this idea was obtained from the results of a pulse-chase experiment that suggested that carnosol accelerated the decay of AR and ER-α in the absence of protein synthesis. Hence, carnosol mediated a decrease in AR and ER-α protein expression seems to be primarily due to a decrease in promoter activity, interference at the LBD, and accelerating the decay of AR and ER-α, leading to a decrease in transactivation and protein stability. This indicates that carnosol could serve as a more effective disruptor of hormone signaling by disrupting AR and ER-α at multiple levels compared with antiandrogens (e.g., flutamide and bicalutamide) or antiestrogens (e.g., tamoxifen or toremifene) that function as antagonists. In our cell-free biochemical-based assays (Fig. 2A and B), the concentration of carnosol was higher than traditional antagonists (e.g., tamoxifen and flutamide); however, this could be explained by the physico-chemical properties of carnosol. Carnosol is a very hydrophobic molecule as evidenced by a high distribution coefficient (logP), suggesting that it is going to favor hydrophobic environments. In a complex biological environment that is primarily made of water, it may not be unreasonable to hypothesize that carnosol will partition into the hydrophobic binding pockets of AR and ER-α. We would like to add that the selected doses of all our in vitro cell culture work performed with carnosol are within the ranges for in vitro work done with other agents that have been evaluated as dual AR and ER-α modulators that include tamoxifen, toremifene, and fulvestrant.

In this study, treatment of LNCaP cells treated with an antiandrogen and antiestrogen led to an increase in AR and ER-α activity that has been observed by others (7, 21–23) and ourselves. This stimulatory effect was reversed when LNCaP cells were cotreated with carnosol (30 μmol/L) and flutamide (30 μmol/L) or tamoxifen (30 μmol/L) with a decrease in AR and ER-α protein expression (Fig. 5C), suggesting that carnosol could reverse the stimulatory action of flutamide and tamoxifen. Interestingly, when LNCaP cells were cotreated with flutamide and tamoxifen, a similar inhibition of AR and ER-α protein expression was observed, suggesting that combination treatment could reverse the stimulatory action of individual treatments. This further indicates that carnosol could serve as a more effective disruptor of AR and ER-α activity compared with flutamide or tamoxifen by inhibiting multiple aspects of hormone signaling.

Although further studies are needed to determine the optimal dose of carnosol in future animal studies, it should be noted that several studies of antiestrogens and antiandrogens have used oral doses up to 100 mg/kg daily. In fact, oral administration of antiandrogens and antiestrogens in animal settings often uses doses that are 166% to 333% higher than the doses of carnosol used in this study (33–36). Given this line of reasoning, future studies comparing carnosol with antiandrogens and antiestrogens should be done using equivalent doses to determine if there is any benefit to increasing the dose of carnosol.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Jeremy J. Johnson, Deeba N. Syed, Yewseok Suh, et al.


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