

Androgen receptor-dependent and -independent mechanisms mediate *Ganoderma lucidum* activities in LNCaP prostate cancer cells

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Abstract. *Ganoderma lucidum* (Curt.:Fr.) P. Karst, a medicinal fungus, has been widely used in Asian countries for centuries to prevent or treat a variety of diseases, including cancer. However, the mechanisms responsible for the effects of *G. lucidum* on cancer cells remain to be elucidated. We have previously shown that ethyl acetate extract of *G. lucidum* inhibits LNCaP prostate cancer cell viability and proliferation. We also demonstrated that *G. lucidum* extract decreased androgen receptor transcriptional activity, suppressed levels of secreted prostate-specific antigen, and suppressed androgen receptor protein level. In this study we investigated the mechanisms that underlie the activities of *G. lucidum* crude extract and its active fraction GLF4 in LNCaP prostate cancer cells. Our data demonstrate that *G. lucidum* inhibits cell viability by induction of apoptosis through the extrinsic pathway that include activation of caspase-8 and caspase-3 and inhibits cell proliferation by the down-regulation of cyclin D1 expression. Furthermore, *G. lucidum* crude extract and fraction GLF4 interfere with androgen receptor function via competition with the natural ligand dihydrotestosterone and suppression of androgen receptor/androgen response element complex formation. These results indicate that *G. lucidum* extracts have profound activity against LNCaP cells that merits further investigation as a potential therapeutic agent for the treatment of prostate cancer.

Introduction

Prostate cancer (PCa) is the most common non-skin malignancy and the third leading cause of cancer deaths worldwide. Prostate

cancer has a complex etiology; presently, age, ethnicity, and family history are the most consistently reported risk factors associated with the disease. Other potential risk factors, such as environmental (1), and dietary (2) factors, have also been suggested. Early-stage prostate cancer that is confined to the prostate gland can be cured by surgical methods (3). In contrast, advanced-stage prostate carcinoma that has metastasized beyond the prostatic capsule is typically treated by androgen-ablation therapies (4). Androgen ablation therapy exploits the androgen-dependency of prostate cancer cells by either reducing endogenous androgen levels that circulate within the body or by directly blocking androgen receptor (AR) activity with chemical inhibitors (5). However, on occasion, cancer cells escape these treatments and reappear as androgen-independent prostate tumors that proliferate in the absence of androgens (6). This class of prostate tumor is often incurable and ultimately leads to the demise of the patient (7). Accumulating evidence support the concept that prostate cancer progression is accompanied by a shift in reliance on endocrine controls to paracrine and eventually autocrine controls and that this complex process is the result of changes, which occur at molecular levels of cellular control (5). However, the molecular mechanisms involved in the development of androgen-independent prostate cancer are unknown. AR gene mutations enable the receptor to be activated by various ligands (other than DHT) and deregulated growth factors and cytokines (usually with the help of AR co-activators); amplified AR can be activated by very low levels of dihydrotestosterin (DHT). In addition, the loss of PTEN reverses the inhibition of the phosphatidylinositol 3-kinase (PI3-K)-Akt pathway, permitting activated Akt to phosphorylate Bad and IKK and consequently to activate the NF- κ B pathway, which eventually leads to inhibition of apoptosis and cell survival (8).

Ganoderma lucidum (Curtis: Fr.) P. Karst, is a woody Basidiomycete fungus belonging to the family of *Ganodermaceae* of Aphyllophorals. It has been widely used in China for the promotion of health and longevity since ancient times. The fungus is a popular health food supplement marketed around the world because of its perceived health benefits, including its anti-cancer properties. In recent years, there has been considerable attention on the anticancer activity

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of *G. lucidum* (9); however, until now, the mechanism of the anticancer action of *G. lucidum* was not well understood and, in the past, activation of the immune response of the host by *G. lucidum* was widely considered as the mechanism responsible for cancer prevention or treatment (10). However, recent studies have shown that in addition to the activation of the immune response of the host, there are other mechanisms by which *G. lucidum* prevents and/or treats cancer. We, for example, showed recently that ethyl acetate extracts from *G. lucidum* inhibits LNCaP cell viability and proliferation, cause a G1 phase arrest, suppress levels of secreted prostate-specific antigen (PSA), and decrease androgen and glucocorticoid receptor transcriptional activity in breast cancer MDA-kb2 cells (11). Furthermore, we were able to identify the active fraction of *G. lucidum*, termed GLF4, which selectively inhibits AR transactivation and competes with DHT for AR binding (Zaidman *et al.*, unpublished data). The present study was undertaken to elucidate the mechanism of action of *G. lucidum* inhibitory activities in LNCaP prostate cancer cells. Here, we demonstrate that *G. lucidum* extract and fraction GLF4, inhibit viability and proliferation of LNCaP cells via induction of apoptosis by activation of caspase-3 via the extrinsic pathway that involves caspase-8, induction of a G1 phase arrest through down-regulation of cyclin D1, and interferes with AR function via competition of *G. lucidum* extracts with the natural ligand DHT and suppression of AR/ARE complex formation.

Materials and methods

Materials. DHT, hydroxyflutamide (OHF), etoposide, and all other fine chemicals were purchased from Sigma-Aldrich (Rehovot, Israel). Tissue culture media and reagents were purchased from Biological Industries (Beit Haemek, Israel). Yeast extract and peptone were purchased from BD Diagnostic Systems (Sparks, MD, USA). *Ganoderma lucidum* (Curt.:Fr.) P. Karst was provided by the Higher Basidiomycetes culture collection at the Institute of Evolution (HAI), University of Haifa, Israel. All antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). [³H]DHT (specific activity 100-150 Ci/mM) was obtained from Amersham (Piscataway, NJ, USA). Caspase-3, -8, and -9 inhibitors Z-DEVD-fmk, Z-IETD-fmk, and Z-LEHD-fmk, respectively, were purchased from EMD Biosciences, Inc. (San Diego, CA, USA).

Fungus culture and extraction. *Ganoderma lucidum* growth conditions have been previously described (11). Dry mycelia were ground to fine powder. The powder was extracted with ethyl acetate (50 ml/g dry mycelia) for 7 days on a rotary shaker at 160 r/min at room temperature. Flask content was paper filtered (Whatman no. 1) and solvents were removed under reduced pressure at a temperature of 40°C. Stock solutions (50 mg/ml) were prepared in DMSO and stored at -70°C. A crude extract was fractionated using a column (65x4.5 cm) packed with silica gel 60 Å, 200-300 mesh. Ethyl acetate extract (2 g) of *G. lucidum* dry mycelia was eluted with a step gradient of n-Hexane-EtOAc-MeOH. Fraction GLF4 was eluted with n-Hexane-EtOAc-MeOH (1.5:1:0 v/v) and yielded 140 mg.

Cell culture. LNCaP prostate cancer (ATCC # CRL-1740) cells were obtained from ATCC (Rockville, MD, USA). Cells were maintained in RPMI-1640 medium containing 2 mM L-glutamine, 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂. Cells were transferred with 0.025% trypsin and 0.02% EDTA. Medium and supplements were obtained from Biological Industries (Beit Haemek, Israel).

Western blot analysis. Cells were plated at 5x10⁵ cells in 25 cm² flasks and after 48 h they were treated with appropriate substances for an additional 48 h. Cells were collected and washed twice in PBS at 1,000 x g for 6 min at 4°C. PBS was removed and cells were lysed in 60 µl of lysis buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 0.1% SDS, and 0.5% sodium deoxycholate, supplemented with protease and phosphatase inhibitor cocktails). Cell lysates were centrifuged at 10,000 x g for 10 min at 4°C, and supernatant was collected. Protein concentrations were determined using a Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (40 µg) were fractionated on 8% SDS-PAGE. The PAGE-fractionated protein extracts were then transferred to nitrocellulose membranes and incubated overnight at 4°C with the appropriate primary antibody. Membranes were washed and incubated with HRP-conjugated secondary antibody. Membranes were incubated with Western blot detection reagents (Bio-Rad, Hercules, CA, USA), and visualized on Kodak BioMax MR film (Sigma-Aldrich).

Measuring caspase-3/7 activity. Cells were plated in white-bottom 96-well plates and incubated for 48 h, then 1 µl of fungal extracts, in different concentrations, was added. Control wells contained combinations of DMSO, 5 nM DHT, or 5 nM DHT plus increasing concentrations of etoposide (apoptosis inducing agent). For measuring caspase 3/7 activity, Caspase-Glo 3/7 (Promega, Madison, WI, USA) assay was used according to the manufacturer's instructions. Briefly, 100 µl of caspase-3/7 reagent was added to each well and plates were incubated for 1 h at RT. Luminescence was determined using Zenyth 3100 microplate luminometer (Anthos Labtec Instruments GmbH, Salzburg, Austria) and quantified as RLU. Activity is defined as the fold induction relative to DHT treatment.

Flow cytometric evaluation of Annexin V-FITC/PI-stained cells. LNCaP cells were plated in 25 cm² flasks and allowed to attach for 48 h. The medium was replaced with 5 ml of phenol-red-free medium supplemented with charcoal-stripped serum, and cells were treated with fungal extracts. Following treatment for 48 h, cells were harvested and pelleted by centrifugation at 1,000 x g for 5 min at room temperature. Cells were washed twice with cold PBS and re-suspended in 1X binding buffer (10 mM HEPES pH 7.5, 140 mM NaCl, 0.25 mM CaCl₂) at a concentration of 1x10⁶ cells/ml. For staining cells, we used Annexin V-FITC Apoptosis Detection Kit, APOAF (Sigma-Aldrich) according to the manufacturer's instructions. Annexin V-FITC (5 µl) and PI (10 µl) were added to 100 µl of cells (1x10⁵ cells).

The tube was vortexed gently and incubated in the dark for 10 min at room temperature. A 1X binding buffer (400 μ l) was added to each tube and the samples analyzed by FACScalibur using Cell Quest software (Becton-Dickinson, San Jose, CA).

Preparation of nuclear extracts from LNCaP cells. Cell fractionation was performed using the CelLytic Nuclear extraction kit (Sigma-Aldrich) according to the manufacturer's instructions. All steps were performed at 4°C, and pre-cooled buffers were used in order to prevent proteolytic degradation of the proteins. The cells were washed twice with 1X PBS and collected in ~600 μ l of ice-cold 1X PBS. The cells were harvested by centrifugation at 400 x g for 3 min at 4°C, and the pellet was re-suspended in 5 packed-cell volumes (PCV) of the hypotonic lysis buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl), containing DTT in a final concentration of 1 mM, and a protease inhibitor cocktail in a final concentration of 1%. The cells were incubated for 15 min on ice allowing them to swell. To swollen cells in hypotonic lysis buffer, 10% IGEPAL CA-630 solution was added to a final concentration of 0.6%. Cells were homogenized by vortexing vigorously for 10 sec. Cells were centrifuged for 30 sec at 10,000 x g. The supernatant (the cytoplasmic fraction) was transferred to fresh tubes and stored at -70°C (after protein concentration determination). The resulting pellet containing 'crude' nuclei was re-suspended in 3 PCV of extraction buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.4 M NaCl, 0.2 mM EDTA (pH 8.0), 25% glycerol] containing DTT in a final concentration of 1 mM, and protease inhibitor cocktail in a final concentration of 1%. Tubes were mounted on a vortex mixer and agitated at medium speed for 30 min. Following centrifugation at 20,000 x g for 5 min, the supernatant (the nuclear fraction) was aliquoted and stored at -70°C.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts from LNCaP cells were used for EMSA studies. DNA binding reactions were carried out with 20 μ g of total protein from nuclear extracts in a total volume of 20 μ l containing 5X binding buffer (1 M Tris pH 7.4, 2.5 M KCl, 0.5 M EDTA pH 8.0, 0.1 M dithiothreitol), 0.5 μ g poly(dI-dC) Amersham (Piscataway, NJ, USA), and 40 ng of double-stranded ARE oligonucleotide (5'-GTCTGGTACAGGG TGTCTTTTT-3') (Panomics, Inc., Fremont, CA, USA). Nuclear extracts and ARE were incubated for 30 min at room temperature and subsequently, protein-DNA complexes were separated under non-denaturing conditions in a 6% polyacrylamide gel (29:1) containing 2.5% glycerol and run in 0.5X TBE (1X = 89 mM Tri-borate, 89 mM boric acid, and 2 mM EDTA pH 8.0) at 80 V at room temperature for 90 min. Then, the gel chamber was dismantled, and the gel was incubated for 20 min in 25 ml 1X TBE containing 2.5 μ l of 10,000X SYBR green (Invitrogen, Carlsbad, CA, USA) nucleic acid gel stain protected from light with gentle agitation. At the end of the incubation period the stain was washed off twice with 150 ml ddH₂O for 10 sec. Digital photographs were taken at 300 nm. The band intensity was measured by densitometry.

Competitive binding of [³H]DHT to the LNCaP AR in the presence of fungal extracts. Competitive binding studies were

performed, with modifications, as described by Long *et al* (12). Twenty-four-well plates were coated with poly-L-lysine (0.05 mg/ml) for 30 min and dried. To determine the kinetics of [³H]DHT-binding to the LNCaP AR, LNCaP cells (2x10⁵) were plated in steroid-free medium and allowed to attach. The following day, the medium was replaced with serum-free, steroid-free RPMI-1640 containing [³H]DHT (0.01-10 nM) in the presence or absence of a 200-fold excess of cold DHT to determine non-specific binding and 1 mM triamcinolone acetonitrile to saturate progesterone and glucocorticoid receptors. Following a 2-h incubation period at 37°C, cells were washed twice with ice-cold PBS and lysed in PBS containing 0.5% SDS and 20% glycerol. Extracts were removed and the cell-associated radioactivity counted in a Tri-carb 2100 TR scintillation counter (Packard Instrument Company, Meriden, CT, USA). The data were analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA), and K_d and B_{max} determined by Scatchard plot transformation. When the concentration of [³H]DHT, required to almost saturate AR in LNCaP cells was established, the ability of the test compounds, in increasing concentrations, to displace [³H]DHT (3.25 nM) from the receptors was determined as described above. Each dilution was performed in triplicate.

Results

Detecting PARP-1 cleavage following fungal extract treatments. One of the key initiation elements of the apoptotic pathway is the activation of caspases (cysteiny-l-aspartic acid proteases) followed by cleavage of the caspase substrates. The 113-kDa poly(ADP-ribose) polymerase 1 (PARP-1) is a nuclear DNA-binding protein normally involved in DNA repair, DNA stability, and other cellular events. Members of the caspase family cleave this protein during early apoptosis. Although PARP is not absolutely required for apoptosis to proceed, the cleavage of PARP may contribute to the commitment to apoptosis. Detection of a 89-kDa or 24-kDa caspase cleavage fragment of PARP-1 was shown to be a hallmark of apoptosis (13). We tested PARP-1 cleavage following treatment with crude organic extract of *G. lucidum* for 24 h. PARP-1 cleavage could be detected following treatment with *G. lucidum* extracts at concentrations of 80 μ g/ml and 120 μ g/ml (Fig. 1), arguing for the ability of *G. lucidum* to induce apoptosis in LNCaP cells. However, treatment with 1 μ M of hydroxyflutamide (OHF) failed to induce PARP-1 cleavage.

Measurement of caspase-3 activity in LNCaP cells treated with *G. lucidum* crude extract and fraction GLF4. To determine if the caspases are involved in fungal extract-induced cell death, LNCaP cells were treated with increasing concentrations of *G. lucidum* crude extract and GLF4 for 24 h. The topoisomerase II inhibitor etoposide served as a positive control. Caspase-3 activity was calculated as the fold increase compared to 5 nM DHT-only treated cells. The results indicate that, at the concentration range tested, *G. lucidum* induced apoptosis in LNCaP in a concentration-dependent manner (Fig. 2A). Caspase-3 activity increased as concentration of *G. lucidum* crude extract and fraction GLF4

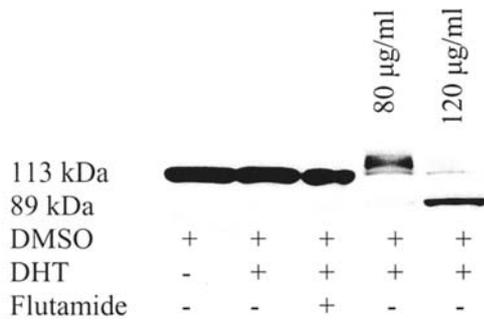
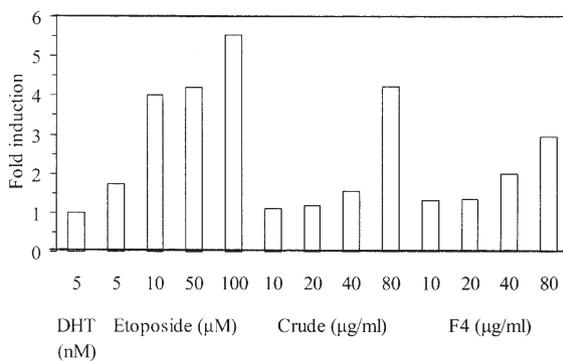


Figure 1. Western blot analysis of PARP-1 cleavage in LNCaP cells. Cells were incubated in phenol-red-free RPMI-1640 containing 10% charcoal/dextran-stripped fetal bovine serum for 48 h followed by treatment with DHT (5 nM) and/or *G. lucidum* crude extract. DMSO (0.5%) and/or hydroxyflutamide (OHF) (1 µM) serve as controls. After 24 h cells were lysed to prepare cellular protein extracts. Aliquots of 40 µg total cellular proteins were separated on 8% SDS gels and transferred to nitrocellulose membranes. PARP cleavage was determined by incubating with mouse anti-human PARP-1 monoclonal antibody (1:2500), followed by a secondary HRP-conjugated goat anti-mouse antibody (1:2500). Immunoreactive signals were detected using a chemiluminescent kit.

A



B

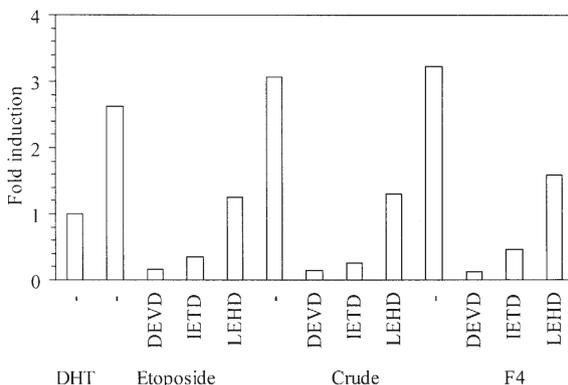


Figure 2. Induction of caspase-3 activity in LNCaP cells by *G. lucidum* crude extract and fraction GLF4. (A) Response to increasing concentrations of inducer. Cells were seeded in a white wall 96-well plate, and allowed to attach. After 48 h cells were treated with increasing concentrations of inducer. Etoposide served as positive control. (B) Effects of caspase inhibitors on etoposide and *G. lucidum*-induced apoptosis. LNCaP cells were treated with 5 nM DHT, 80 µg/ml *G. lucidum* crude extract, or 40 µg/ml fraction GLF4 for 48 h. Five µM caspase-3 inhibitor (Z-DEVD-fmk), caspase-8 inhibitor (Z-IETD-fmk) and caspase-9 inhibitor (Z-LEHD-fmk) were added 2 h prior to treatment with etoposide or *G. lucidum* extracts. In both experiments caspase-3 reagent was added and plates were incubated for 1 h. Levels of caspase-3 were monitored using luminometer and quantified as RLU. Fold induction in caspase-3 activity was calculated against DHT-treated cells.

increased, reaching a maximum level at a concentration of 80 µg/ml. Crude extract at 80 µg/ml was more active by 30% in inducing caspase-3 activity as compared to fraction GLF4 (Fig. 2A). In addition, topoisomerase II inhibitor etoposide significantly induced caspase-3 activity in a concentration-dependent manner.

To identify which apical caspase (caspase-8 or -9) is involved in the initiation of apoptosis induced by *G. lucidum* extract and fraction GLF4, we used the caspase-3, -8, and -9 inhibitors Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK, respectively. With no inhibitor added, the anti-cancer drug etoposide increased caspase-3 activity by 2.6-fold as compared to DHT-treated cells (Fig. 2B). *G. lucidum* crude extract and fraction GLF4 induced caspase-3 activity comparable with the effect of etoposide, increasing caspase-3 activity 3- and 3.2-fold, respectively, over DHT-treated cells (Fig. 2B). When cells were cultured with fungal extracts and the inhibitors Z-DEVD-fmk or Z-IETD-fmk, apoptosis induction was significantly reduced (Fig. 2B). However, when cells were pre-treated with inhibitor Z-LEHD-fmk, *G. lucidum* crude extract and fraction GLF4 increased caspase-3 activity 1.3- and 1.6-fold, respectively, over DHT-treated cells (Fig. 2B). These results demonstrate that inhibitors of caspase-3 and -8 abrogated caspase-3 activity induced by *G. lucidum* crude extract and fraction GLF4. However, inhibitor of caspase-9 was less efficient than the other inhibitors used. Thus, caspase-3 and -8 are involved in the apoptosis-inducing function of *G. lucidum* crude extract and fraction GLF4.

Annexin V binding and PI influx in LNCaP cells treated with G. lucidum crude extract and fraction GLF4. To corroborate our previous data showing apoptosis-inducing ability of *G. lucidum* extract and fraction GLF4, we analyzed the transmigration of PS residues from the inner to outer plasma membrane leaflet - characteristic of programmed cell death. *G. lucidum* crude extract increased the percentage of annexin V-FITC-positive cells 3.2-, 3.3-, and 4.7-fold in cells treated with 10, 40, and 160 µg/ml *G. lucidum* crude extract, respectively, compared to control cells (Fig. 3A). *G. lucidum* fraction GLF4 increased the percentage of annexin V-FITC-positive cells 3.0-, 3.4-, and 5.2-fold in cells treated with 10, 40, and 160 µg/ml *G. lucidum* fraction GLF4, respectively, compared to control cells (Fig. 3B). There was no significant difference between *G. lucidum* crude extract and fraction GLF4 treatments on annexin V-FITC-positive cells at either concentration. PI staining was significantly lower in *G. lucidum* crude extract and fraction GLF4 treated cells in comparison to annexin V-FITC staining indicating a lack of marked necrosis or increased plasma membrane permeability at the concentration range tested (Fig. 3). There was no significant difference between *G. lucidum* crude extract and fraction GLF4 treatments on PI-positive cells at either concentration.

G. lucidum crude extract and fraction GLF4 down-regulate cyclin D1. We previously showed (11) that in LNCaP cells treated with *G. lucidum* extracts, there is an increase in the percentage of the cell population in the G1 phase accompanied with a decrease in the percentage of cell population in the S phase; an observation that was consistent with a G1

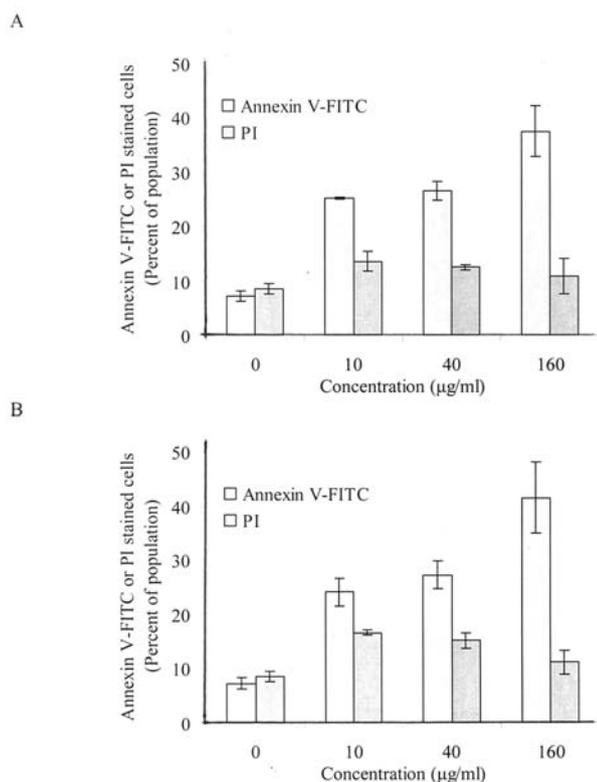


Figure 3. Annexin V-FITC binding and PI influx in LNCaP cells. Cells were incubated for 48 h with DHT alone or with DHT and increasing concentrations of *G. lucidum* crude extract (A) and fraction GLF4 (B). Stained cells were analyzed by FACS. Data represent means of 2 replicates and indicate the percentage of cells staining positively for annexin V-FITC or PI. Bars indicate standard deviation.

phase arrest of the cell cycle. To elucidate the mechanisms of *G. lucidum* crude extract and fraction GLF4-induced cell cycle arrest in LNCaP cells, we evaluated cyclin D1 expression in LNCaP treated cells by Western blotting. As shown in Fig. 4, both the crude extract and fraction GLF4 markedly decreased the expression of cyclin D1.

Analysis of AR cytoplasmic/nuclear distribution in LNCaP cells treated with *G. lucidum* crude extract and fraction GLF4.

To examine the effect of *G. lucidum* extracts on nuclear translocation of the AR, a Western blot analysis was conducted. LNCaP cells were treated with *G. lucidum* extracts in the presence and absence of 5 nM DHT. Cytoplasmic and nuclear protein fractions were extracted and subjected to Western blot analysis for the AR. DHT treatment cause the accumulation of AR in the nucleus, however, presence of hydroxyflutamide (OHF) alongside DHT prevented nuclear accumulation of AR. In addition, treatment with *G. lucidum* crude extract prevented nuclear accumulation in a dose-dependent manner and consequently cytosolic AR levels were increased. Interestingly, at higher *G. lucidum* crude extract concentration (160 µg/ml) AR was not detected in either fraction (Fig. 5A). Although activity of *G. lucidum* crude extract had a much more pronounced effect on nuclear translocation than fraction GLF4, fraction GLF4 also inhibited AR nuclear translocation. At the highest concentration used (160 µg/ml) AR was mainly present in

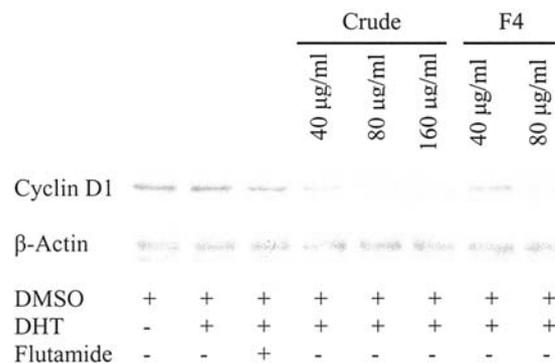


Figure 4. Effect of *G. lucidum* crude extract and fraction GLF4 on expression of cyclin D1 protein. Cells were treated with increasing concentrations of *G. lucidum* extracts for 24 h. Total cellular proteins were prepared and Western blotting was performed with an antibody specific for corresponding proteins.

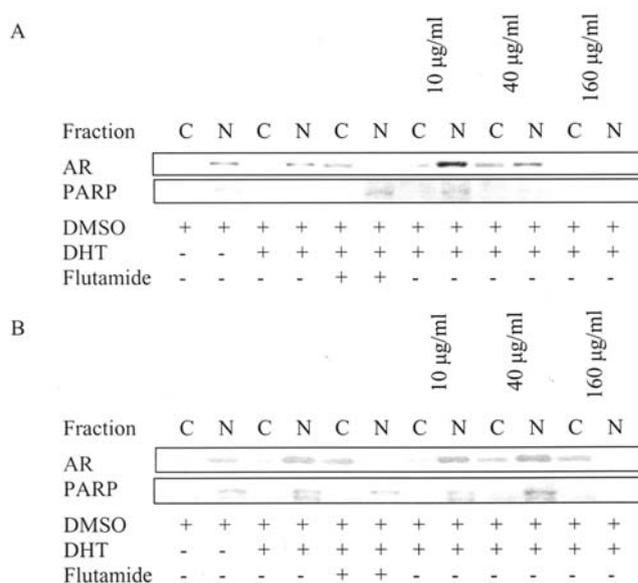


Figure 5. Cellular localization of the AR in LNCaP cells. Effects of *G. lucidum* crude extract (A) and fraction GLF4 (B) on AR localization. Cells were incubated in phenol-red-free RPMI-1640 containing 10% charcoal/dextran-stripped fetal bovine serum for 48 h followed by transferring the cells to 1% charcoal/dextran-stripped fetal bovine serum for an additional 24 h. Cells were treated with DHT (5 nM) and/or fungal extracts. DMSO (0.5%) and/or hydroxyflutamide (OHF) (5 µM) serve as controls. After 2-h treatment cytosolic and nuclear proteins were extracted. Western blot analysis was performed using antibodies to the AR and PARP. PARP serves as an indicator for nuclear fractions.

the cytoplasmic fraction and absent in the nuclear fraction (Fig. 5B).

DNA binding activity of *G. lucidum* crude extract and fraction GLF4. To explore the effect of *G. lucidum* extract and fraction GLF4 on binding of AR to ARE, EMSA was employed. The complex formation was observed using the consensus region of ARE and nuclear extracts from LNCaP cells. The treatment of LNCaP cells with DHT (5 nM) enhanced AR/ARE complex formation as compared to non-activated AR (Fig. 6). Hydroxyflutamide (5 µM), as a positive control, moderately

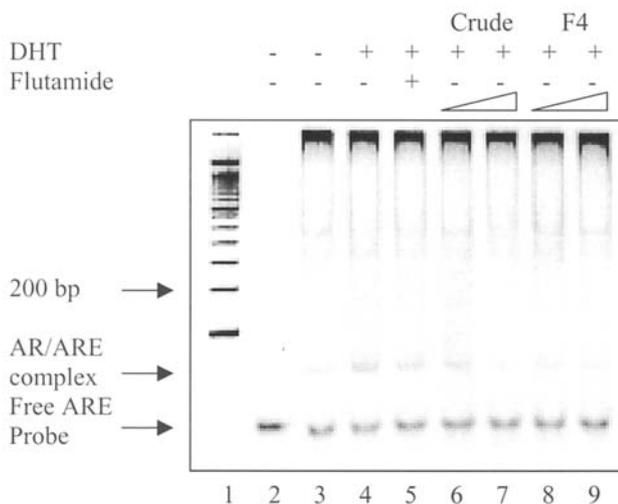


Figure 6. Gel mobility shift analysis of the AR. A 31-bp oligonucleotide consisting of AR responsive element was used for this analysis. Twenty micrograms of nuclear extracts from LNCaP cells were mixed with 0.5 µg poly(dI·dC) and 40 ng DNA fragment and incubated for 20 min on ice. Samples were analyzed by electrophoresis on native 6% polyacrylamide gels at 16 V/cm for 2 h at RT. The first lane contains 100-bp DNA ladder. The second lane contains a probe with no nuclear extract. Cells were treated with *G. lucidum* crude extract at 40 and 120 µg/ml (lanes 6 and 7) and fraction GLF4 at 40 and 120 µg/ml (lanes 8 and 9).

Similarly, *G. lucidum* fraction GLF4 at concentrations 40 and 120 µg/ml significantly suppressed the AR/ARE complex formation (Fig. 6). These results show that *G. lucidum* crude extract and fraction GLF4 inhibited binding of DHT-activated AR to the ARE.

G. lucidum crude extract and fraction GLF4 compete with androgen for binding to the AR in LNCaP cells. Since our results strongly implicate the AR as a central factor in mediating the activity of *G. lucidum* crude extract and fraction GLF4 in LNCaP cells, we directly assessed the ability of crude extract and fraction GLF4 to bind to this receptor. As expected, cold DHT and the well-known anti-androgen hydroxyflutamide were able to displace the radioligand with EC₅₀ values of 11.93±0.13 nM, and 0.11±0.057 µM, respectively (Fig. 7A). Results from competitive binding assays with the mutant AR of LNCaP cells demonstrate that *G. lucidum* crude extract and fraction GLF4, in the concentration range tested, compete with labeled DHT for binding to the AR (Fig. 7B). Fraction GLF4 has higher binding affinity than the crude extract as is evidenced by three orders of magnitude lower values of EC₅₀ (168.7 µg/ml for the crude extract and 0.178 µg/ml for GLF4).

Discussion

Ganoderma is a Basidiomycete white rot fungus that has been used for medicinal purposes for centuries - particularly in China, Japan, and Korea. Most of the scientific research has been performed on the species, *G. lucidum*. Paterson (9) nicknamed this fungus 'a therapeutic fungal bio-factory'. Indeed, this fungus produces anti-tumor and hypoglycemic polysaccharides (10), immunomodulatory protein (14), and many bioactive oxygenated triterpenoids. Up to now, over 130 species of triterpenoids and sterols have been isolated from *G. lucidum* and the genus *Ganoderma* (15). We have previously reported that *G. lucidum* crude extract inhibits LNCaP cell viability and proliferation, and causes a G1 phase arrest in LNCaP, but not in DU 145 and PC-3 cells. We also demonstrated that *G. lucidum* decreased androgen and glucocorticoid receptor transcriptional activity in breast cancer MDA-kb2 cells, suppressed levels of secreted PSA, and suppressed androgen receptor protein levels in LNCaP (11). Furthermore, we were able to isolate an active fraction from *G. lucidum*, termed GLF4, which selectively interfered with AR activity, but not with GR in MDA-kb2 cells and, consequently, inhibited PSA secretion in LNCaP treated cells (Zaidman *et al.*, unpublished data).

In the present study, we examined the mechanisms that underlie the effects of *G. lucidum* in LNCaP prostate cancer cells. Here, we show that *G. lucidum* crude extract and fraction GLF4 inhibit cell viability by inducing apoptosis through the extrinsic pathway that include activation of caspase-8 and caspase-3, and inhibiting cell proliferation by the down-regulation of cyclin D1 expression. Furthermore, *G. lucidum* interfere with the androgen receptor function via competition of compounds present in *G. lucidum* extracts with the natural ligand DHT and with suppression of AR/ARE complex formation.

We measured three parameters that indicate induction of apoptosis in LNCaP cells: PARP cleavage (Fig. 1), caspase-3

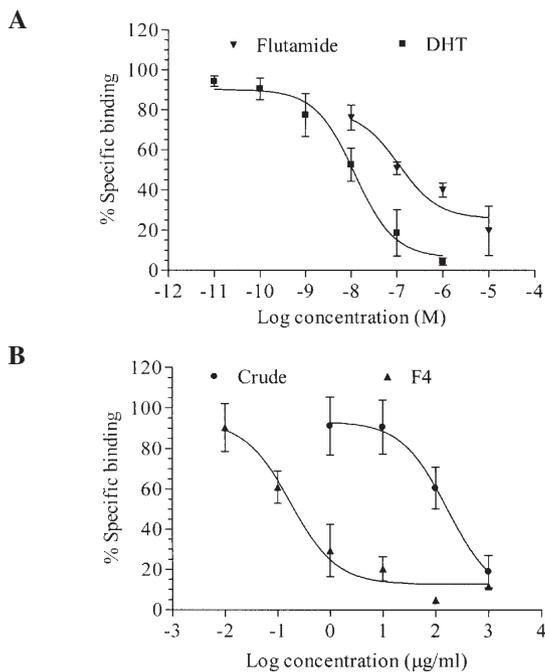


Figure 7. Competitive inhibition of [³H]DHT binding to the AR by *G. lucidum* crude extract and fraction GLF4. LNCaP cells were incubated with increasing concentrations of each fungal extract in the presence of 3.25 nM [³H]DHT. Whole-cell binding assay was performed with (A) DHT, hydroxyflutamide (OHF) as positive control; and *G. lucidum* crude extract and fraction GLF4 (B). Data are expressed as percentages of the specific binding observed in the non-competing control. The values represent the means ± SD of two replicates.

inhibited the DHT-stimulated complex formation (Fig. 6). *G. lucidum* crude extract at concentrations of 40 and 120 µg/ml significantly suppressed the AR/ARE complex formation.

activity (Fig. 2), and annexin V-FITC staining of externalized PS membranes (Fig. 3). Collectively, these results indicated that *G. lucidum* extract and fraction GLF4 induce apoptosis by triggering the caspase cascade through the extrinsic or death receptor mediated pathway that includes the 'initiator' caspase-8, and the 'effector' caspase-3. The ability of *G. lucidum* extracts to induce apoptosis was demonstrated in several cell lines, e.g., human leukemia, lymphoma and multiple myeloma (16), human breast cancer MCF-7 (17), human prostate cancer PC-3 (18), human hepatoma HuH-7 (19), and human colonic carcinoma HT-29 (20) cell lines. It was shown that a mixture of extracts from *G. lucidum* and the herb *Duchesnea chrysantha* induce apoptosis in human leukemia HL-60 cells (21). However, as opposed to our findings, in some of these cell lines it was found that *G. lucidum* extracts induce apoptosis through the intrinsic or mitochondrial pathway (17,19,21). Apoptosis is the predominant mechanism by which cancer cells die when subjected to chemotherapy or irradiation. However, cancer cells develop resistance to these therapies that may be due, at least in part, to the development of effective anti-apoptotic mechanisms. For example, in PCa cells there is increased expression of anti-apoptotic proteins of the Bcl-2 family, e.g., Bcl-2, Bcl-xL, and Mcl-1 (22). Another mechanism allowing escape from apoptosis is activation of survival signal transduction pathways. Several survival signaling pathways have been described, some of them are Akt-dependent (23), and others are not (24). The best characterized pathway is signaling via activation of PI3K and the protein kinase Akt. Activated Akt protects cells from apoptotic death by phosphorylating substrates such as BAD, procaspase-9, or substrates such as the human telomerase reverse transcriptase subunit, forkhead transcription family members, or I κ B kinases that indirectly inhibit apoptosis. In LNCaP PCa cells, Akt is constitutively active as a result of a frame-shift mutation in the *PTEN* tumor suppressor gene, which encodes a phosphatase that inactivates the lipid products of PI3K, and, consequently, the NF- κ B pathway is constitutively activated (25). In a series of experiments, Sliva *et al* (26,27) and Jiang *et al* (18,28) showed that *G. lucidum* extracts inhibit viability, proliferation, and induce apoptosis, which is mediated in part by the inhibition of the Akt/NF- κ B signaling in PCa PC-3 and breast cancer MDA-MD-231 cells. The possibility to bypass survival signals makes pro-apoptotic signaling via death receptors a promising tool for therapeutic intervention in PCa cells, hence, the advantage of *G. lucidum* extracts.

We have previously shown that *G. lucidum* fungal extracts blocked LNCaP cell cycle at the transition from G1 to S phase. These results are in accordance with several recent reports showing a G1 phase arrest (17,18,28-30). Interestingly, there are reports indicating a G2 phase arrest (18,31,32) following treatment with *G. lucidum*. In this report we demonstrate that the G1 phase arrest is mediated through down-regulation of cyclin D1. These results are in accordance with other studies demonstrating down-regulation of cyclin D1 accompanied with G1 phase arrest (17,28).

Mammalian cell growth and proliferation are mediated via cell cycle progression (33). Progression through the G1 phase of the cell division cycle is a rate-limiting step in

mammalian cell proliferation and is governed by numerous mitogenic pathways until the restriction point is passed. CDK4 and CDK6 complexed with cyclin D1 are responsible for cell cycle progression through the G1 phase (34), and the CDK2/cyclin E complex functions in the progression of the cell from late G1 to early S phase (35). These complexes lead to the phosphorylation of retinoblastoma gene product, a tumor suppressor gene that is active in controlling the G1 phase (36). Hyper-phosphorylated pRb leads to its release from the E2F family of transcription factors and induces expression of a number of genes required for S-phase transition (37). We hypothesize, therefore, that down-regulation of cyclin D1 expression caused by *G. lucidum* will lead to dephosphorylation of Rb and, consequently, to growth arrest.

The model of AR activation includes several sequential steps. The AR is known to exist as a monomer in the cytoplasm of responsive cells, sequestered in a complex with heat-shock proteins (HSPs). On administration and binding of androgens, the AR changes conformation, and the HSPs are released, followed by a second conformational change. The receptor dimerizes, binds to DNA, and phosphorylation occurs. The DNA-bound receptor interacts with the transcription initiation complex to regulate transcription of genes involved in growth and survival of PCa. We have demonstrated previously that *G. lucidum* decrease transcriptional activity of the AR (11).

In this report we show that treatment of LNCaP cells with DHT alone induced nuclear translocation of the AR and that co-treatment with *G. lucidum* crude extract inhibited DHT-induced translocation in a concentration-dependent manner, retaining the AR in the cytoplasm (Fig. 5A). Co-treatment with fraction GLF4 had an intriguing effect; retaining the AR in the cytoplasm, while levels in the nucleus hardly changed (Fig. 5B). In both treatments it appears that the highest concentration used was toxic to the cells. Moreover, in cells treated with the non-steroidal anti-androgen hydroxyflutamide, most AR was found in the cytoplasmic fraction with very small amounts in the nuclear fraction.

There are conflicting reports regarding the effect of some anti-androgens on AR nuclear import. Early reports demonstrated that the steroidal (e.g., cyproterone acetate) and non-steroidal anti-androgens (e.g., OHF and Bicalutamide) induce nuclear localization at varying degrees (38,39). A more recent study employed an elaborate fractionation of the cell into five distinct subcellular fractions (40). In this study it was shown that in cells treated with OHF and Bicalutamide there was a reversible retention of the AR to the nuclear matrix, whereas in cells treated with cyproterone acetate there is an association of the AR with cytoplasmic membranes and an irreversible retention within the cytoplasm. Both of these anti-androgen groups effectively inhibited transcriptional activity of the AR (41).

Thus, it seems that there are several compounds present in *G. lucidum* extracts that affect transcriptional activity of the AR. Both *G. lucidum* crude extract and fraction GLF4 compete effectively with DHT on binding to the AR as was revealed in the competitive binding assay (Fig. 7B). The average binding affinity of fraction GLF4 compounds is much higher than that of crude extract compounds (Fig. 7B).

Moreover, both the crude extract and fraction GLF4 are able to inhibit DHT-activated AR to ARE as measured by EMSA (Fig. 6). In the highest concentration tested, reduction in DNA binding was almost the same for both fractions. These results imply different mechanisms of action for both the crude extract and fraction GLF4. Our working hypothesis is that *G. lucidum* crude extract contains substances that compete with DHT on binding to the AR, decrease nuclear localization and decrease DNA binding in a concentration-dependent manner, thus decreasing transcriptional activity. Fraction GLF4 compounds on the other hand, maintain high levels of AR in the nucleus and decrease DNA binding to a constant level regardless of the concentration used. We suggest, therefore, that fraction GLF4, like other anti-androgens, stimulates the assembly of a transcriptionally inactive AR on DNA (42) possibly through inhibition of the interaction with co-activators and/or enhancement of the interaction with co-repressors (43). Thus, some of the substances that regulate AR function, probably by ligand competition, and affect LNCaP cell viability are present in GLF4. However, other compounds that are present in the crude extract and are missing in GLF4 could possibly affect AR indirectly or decrease viability and proliferation of LNCaP cells by different mechanisms.

It is of great interest for us to purify and elucidate the chemical structure of active moieties from *G. lucidum* fraction GLF4. In addition, we are interested in exploiting the natural diversity of the active moieties in quantity and composition found in different species of the same genus and within strains of the selected species.

In conclusion, our data demonstrated that AR-dependent and independent mechanisms underlie the inhibitory activities of *G. lucidum* extracts in LNCaP prostate cancer cells. Furthermore, our data revealed the possibilities of utilizing *G. lucidum* in a drug discovery effort aimed at developing bioactive metabolites from *G. lucidum* that could yield an anti-prostate cancer therapy.

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References

- Hayes RB: Gene-environment interrelations in prostate cancer. *Epidemiol Rev* 23: 163-167, 2001.
- Baillargeon J and Rose DP: Obesity, adipokines, and prostate cancer (review). *Int J Oncol* 28: 737-745, 2006.
- Naito S: Evaluation and management of prostate-specific antigen recurrence after radical prostatectomy for localized prostate cancer. *Jpn J Clin Oncol* 35: 365-374, 2005.
- Miles BJ and Babiarz J: Maximal androgen ablation: a review. *Henry Ford Hosp Med J* 40: 114-117, 1992.
- Singh P, Uzgar A, Litvinov I, Denmeade SR and Isaacs JT: Combinatorial androgen receptor targeted therapy for prostate cancer. *Endocr Relat Cancer* 13: 653-666, 2006.
- Wright ME, Tsai MJ and Aebersold R: Androgen receptor represses the neuroendocrine transdifferentiation process in prostate cancer cells. *Mol Endocrinol* 17: 1726-1737, 2003.
- Hsieh WS and Simons JW: Systemic therapy of prostate cancer. New concepts from prostate cancer tumor biology. *Cancer Treat Rev* 19: 229-260, 1993.
- Debes JD and Tindall DJ: Mechanisms of androgen-refractory prostate cancer. *N Engl J Med* 351: 1488-1490, 2004.
- Paterson RR: *Ganoderma* - a therapeutic fungal biofactory. *Phytochemistry* 67: 1985-2001, 2006.
- Lin ZB: Cellular and molecular mechanisms of immunomodulation by *Ganoderma lucidum*. *J Pharmacol Sci* 99: 144-153, 2005.
- Zaidman BZ, Wasser SP, Nevo E and Mahajna J: *Coprinus comatus* and *Ganoderma lucidum* interfere with androgen receptor function in LNCaP prostate cancer cells. *Mol Biol Rep* published online: 13 March 2007 DOI: 10.1007/s11033-007-9059-5.
- Long BJ, Grigoryev DN, Nnane IP, Liu Y, Ling YZ and Brodie AM: Antiandrogenic effects of novel androgen synthesis inhibitors on hormone-dependent prostate cancer. *Cancer Res* 60: 6630-6640, 2000.
- Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG and Earnshaw WC: Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371: 346-347, 1994.
- Lin WH, Hung CH, Hsu CI and Lin JY: Dimerization of the N-terminal amphipathic alpha-helix domain of the fungal immunomodulatory protein from *Ganoderma tsugae* (Fip-gts) defined by a yeast two-hybrid system and site-directed mutagenesis. *J Biol Chem* 272: 20044-20048, 1997.
- Gao JJ, Nakamura N, Min BS, Hirakawa A, Zuo F and Hattori M: Quantitative determination of bitter principles in specimens of *Ganoderma lucidum* using high-performance liquid chromatography and its application to the evaluation of ganoderma products. *Chem Pharm Bull* 52: 688-695, 2004.
- Muller CI, Kumagai T, O'Kelly J, Seeram NP, Heber D and Koeffler HP: *Ganoderma lucidum* causes apoptosis in leukemia, lymphoma and multiple myeloma cells. *Leuk Res* 30: 841-848, 2006.
- Hu H, Ahn NS, Yang X, Lee YS and Kang KS: *Ganoderma lucidum* extract induces cell cycle arrest and apoptosis in MCF-7 human breast cancer cell. *Int J Cancer* 102: 250-253, 2002.
- Jiang J, Slivova V, Valachovicova T, Harvey K and Sliva D: *Ganoderma lucidum* inhibits proliferation and induces apoptosis in human prostate cancer cells PC-3. *Int J Oncol* 24: 1093-1099, 2004.
- Li CH, Chen PY, Chang UM, *et al*: Ganoderic acid X, a lanostanoid triterpene, inhibits topoisomerases and induces apoptosis of cancer cells. *Life Sci* 77: 252-265, 2005.
- Hong KJ, Dunn DM, Shen CL and Pence BC: Effects of *Ganoderma lucidum* on apoptotic and anti-inflammatory function in HT-29 human colonic carcinoma cells. *Phytother Res* 18: 768-770, 2004.
- Kim KC, Kim JS, Son JK and Kim IG: Enhanced induction of mitochondrial damage and apoptosis in human leukemia HL-60 cells by the *Ganoderma lucidum* and *Duchesnea chrysantha* extracts. *Cancer Lett* 246: 210-217, 2007.
- Bruckheimer EM, Spurgers K, Weigel NL, Logothetis C and McDonnell TJ: Regulation of Bcl-2 expression by dihydrotestosterone in hormone sensitive LNCaP-FGC prostate cancer cells. *J Urol* 169: 1553-1557, 2003.
- Murillo H, Huang H, Schmidt LJ, Smith DI and Tindall DJ: Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. *Endocrinology* 142: 4795-4805, 2001.
- Li P, Lee H, Guo S, Unterman TG, Jenster G and Bai W: AKT-independent protection of prostate cancer cells from apoptosis mediated through complex formation between the androgen receptor and FKHR. *Mol Cell Biol* 23: 104-118, 2003.
- Deeb D, Jiang H, Gao X, *et al*: Curcumin sensitizes prostate cancer cells to tumor necrosis factor-related apoptosis-inducing ligand/Apo2L by inhibiting nuclear factor-kappaB through suppression of IkkappaBalpha phosphorylation. *Mol Cancer Ther* 3: 803-812, 2004.
- Sliva D, Labarrere C, Slivova V, Sedlak M, Lloyd FP Jr and Ho NW: *Ganoderma lucidum* suppresses motility of highly invasive breast and prostate cancer cells. *Biochem Biophys Res Commun* 298: 603-612, 2002.
- Sliva D, Sedlak M, Slivova V, Valachovicova T, Lloyd FP Jr and Ho NW: Biologic activity of spores and dried powder from *Ganoderma lucidum* for the inhibition of highly invasive human breast and prostate cancer cells. *J Altern Complement Med* 9: 491-497, 2003.
- Jiang J, Slivova V, Harvey K, Valachovicova T and Sliva D: *Ganoderma lucidum* suppresses growth of breast cancer cells through the inhibition of Akt/NF-kappaB signaling. *Nutr Cancer* 49: 209-216, 2004.

29. Zhu HS, Yang XL, Wang LB, Zhao DX and Chen L: Effects of extracts from sporoderm-broken spores of *Ganoderma lucidum* on HeLa cells. *Cell Biol Toxicol* 16: 201-206, 2000.
30. Yang HL: Ganoderic acid produced from submerged culture of *Ganoderma lucidum* induces cell cycle arrest and cytotoxicity in human hepatoma cell line BEL7402. *Biotechnol Lett* 27: 835-838, 2005.
31. Lin SB, Li CH, Lee SS and Kan LS: Triterpene-enriched extracts from *Ganoderma lucidum* inhibit growth of hepatoma cells via suppressing protein kinase C, activating mitogen-activated protein kinases and G2-phase cell cycle arrest. *Life Sci* 72: 2381-2390, 2003.
32. Lu QY, Jin YS, Zhang Q, *et al*: *Ganoderma lucidum* extracts inhibit growth and induce actin polymerization in bladder cancer cells *in vitro*. *Cancer Lett* 216: 9-20, 2004.
33. Ni J, Chen M, Zhang Y, Li R, Huang J and Yeh S: Vitamin E succinate inhibits human prostate cancer cell growth via modulating cell cycle regulatory machinery. *Biochem Biophys Res Commun* 300: 357-363, 2003.
34. Fu M, Wang C, Li Z, Sakamaki T and Pestell RG: Minireview: Cyclin D1: normal and abnormal functions. *Endocrinology* 145: 5439-5447, 2004.
35. Angus SP, Fribourg AF, Markey MP, *et al*: Active RB elicits late G1/S inhibition. *Exp Cell Res* 276: 201-213, 2002.
36. Xu Y, Chen SY, Ross KN and Balk SP: Androgens induce prostate cancer cell proliferation through mammalian target of rapamycin activation and post-transcriptional increases in cyclin D proteins. *Cancer Res* 66: 7783-7792, 2006.
37. Hofman K, Swinnen JV, Verhoeven G and Heyns W: E2F activity is biphasically regulated by androgens in LNCaP cells. *Biochem Biophys Res Commun* 283: 97-101, 2001.
38. Kemppainen JA, Lane MV, Sar M and Wilson EM: Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and anti-hormones. *J Biol Chem* 267: 968-974, 1992.
39. Jenster G, Trapman J and Brinkmann AO: Nuclear import of the human androgen receptor. *Biochem J* 293: 761-768, 1993.
40. Whitaker HC, Hanrahan S, Totty N, *et al*: Androgen receptor is targeted to distinct subcellular compartments in response to different therapeutic antiandrogens. *Clin Cancer Res* 10: 7392-7401, 2004.
41. Tyagi RK, Lavrovsky Y, Ahn SC, Song CS, Chatterjee B and Roy AK: Dynamics of intracellular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells. *Mol Endocrinol* 14: 1162-1174, 2000.
42. Masiello D, Cheng S, Bubley GJ, Lu ML and Balk SP: Bicalutamide functions as an androgen receptor antagonist by assembly of a transcriptionally inactive receptor. *J Biol Chem* 277: 26321-26326, 2002.
43. Song LN, Coghlan M and Gelmann EP: Antiandrogen effects of mifepristone on coactivator and corepressor interactions with the androgen receptor. *Mol Endocrinol* 18: 70-85, 2004.