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 transcripational 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 dihydrotestosterin 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-κB 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 Aphyllorals. 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...
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 Haemeq, 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 Basidimycetes 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). ['H]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. NaCl, 0.25 mM CaCl2) at a concentration of 1x106 cells/ml. 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 resuspended in 1X binding buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na3P2O7, 2 mM Na3VO4, 1% Triton X-100, 10% glycerol, 1 mPMFS, 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).

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 resuspended 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 (1x106 cells).
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 μl 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 μg of total protein from nuclear extracts in a total volume of 20 μl 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) 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.

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 (2x10^5) 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 acetonitride 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 Kd and Bmax 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 (cysteiny1-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 μg/ml and 120 μg/ml (Fig. 1), arguing for the ability of G. lucidum to induce apoptosis in LNCaP cells. However, treatment with 1 μM 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 mM 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
were detected using a chemiluminescent kit. HRP-conjugated goat anti-mouse antibody (1:2500). Immunoreactive signals were quantified as RLU. Fold induction in caspase-3 activity was calculated against DHT-treated cells.

Figure 2. Induction of caspase-3 activity in LNCaP cells by DHT (5 nM) and/or dextran-stripped fetal bovine serum for 48 h followed by treatment with 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 antihuman 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.

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 (OHH) (1 μM) serve as controls. After 24 h cells were lysed to prepare cellular protein extracts. Aliquots of 40 μg total cellular protein extracts 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.

...induced apoptosis. In both experiments caspase-3 reagent was added and plates were incubated for 1 h. Levels of caspase-3 were monitored using luminometer and (Z-LEHD-fmk) were added 2 h prior to treatment with etoposide or inhibitors Z-DEVD-fmk, Z-IETD-fmk, and Z-LEHD-fmk, respectively. When cells were pre-treated with inhibitor Z-LEHD-fmk, 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 transmembrane 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 crude extract, 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 treatments on annexin V-FITC-positive cells at either concentration. PI staining was significantly lower in 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 treatments on annexin V-FITC-positive cells at either concentration. PI staining was significantly lower in 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 treatments on annexin V-FITC-positive cells at either concentration. PI staining was significantly lower in G. lucidum crude extract and fraction GLF4 treatments on annexin V-FITC-positive cells at either concentration. 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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
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 caused 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 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
inhibited the DHT-stimulated complex formation (Fig. 6). It was found that 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
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 IκB 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-κB pathway is constitutively activated (25). In a series of experiments, Silva 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-κB 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 exploring 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


