Ganoderma lucidum Suppresses Growth of Breast Cancer Cells Through the Inhibition of Akt/NF-κB Signaling

Jiahua Jiang, Veronika Slivova, Kevin Harvey, Tatiana Valachovicova, and Daniel Sliva

Abstract: Ganoderma lucidum (Reishi, Lingzhi) is a popular Asian mushroom that has been used for more than 2 millennia for the general promotion of health and was therefore called the “Mushroom of Immortality.” Ganoderma lucidum was also used in traditional Chinese medicine to prevent or treat a variety of diseases, including cancer. We previously demonstrated that Ganoderma lucidum suppresses the invasive behavior of breast cancer cells by inhibiting the transcription factor NF-κB. However, the molecular mechanisms responsible for the inhibitory effects of Ganoderma lucidum on the growth of highly invasive and metastatic breast cancer cells has not been fully elucidated. Here, we show that Ganoderma lucidum inhibits proliferation of breast cancer MDA-MB-231 cells by downregulating Akt/NF-κB signaling. Ganoderma lucidum suppresses phosphorylation of Akt on Ser\(^{373}\) and downregulates the expression of Akt, which results in the inhibition of NF-κB activity in MDA-MB-231 cells. The biological effect of Ganoderma lucidum was demonstrated by cell cycle arrest at G0/G1, which was the result of the downregulation of expression of NF-κB-regulated cyclin D1, followed by the inhibition of cdk4. Our results suggest that Ganoderma lucidum inhibits the growth of MDA-MB-231 breast cancer cells by modulating Akt/NF-κB signaling and could have potential therapeutic use for the treatment of breast cancer.

Introduction

One third of all newly diagnosed cancers among women in the United States are breast cancers (1). Because breast cancer often progresses from the therapy-responsive phenotype to the highly invasive and metastatic phenotype, breast cancer is the second leading cause of cancer death in the U.S. female population (2). A comprehensive review by the World Cancer Research Fund and the American Institute of Cancer Research clearly demonstrates the importance of nutrition in the prevention of cancer, which could also contribute to the low incidence of breast cancers among Asian women (3). Therefore, it was suggested that some nutritional products have chemopreventive and therapeutic effects against cancer. These anticancer effects also significantly increased the popularity of dietary supplements in cancer patients (4).

The popular edible mushroom *Ganoderma lucidum* has been widely used in eastern Asia to promote health and longevity. The regular consumption of *Ganoderma lucidum* in the form of teas was believed to fortify the mind and the body, and therefore *Ganoderma lucidum* was called the “Mushroom of Immortality” (5). The dried powder of *Ganoderma lucidum* has been used in traditional Chinese medicine for more than 2,000 years to prevent or treat different diseases, including cancer (6). The anticancer properties of *Ganoderma lucidum* have been attributed to either the isolated polysaccharides, which are responsible for the stimulation of the immune system, or triterpenes, which demonstrate cytotoxic activity against cancer cells (for review, see ref. 7). However, *Ganoderma lucidum* is currently available as a dietary supplement in the form of extracts or capsules containing fruiting bodies and/or spores of this mushroom. We recently reported that *Ganoderma lucidum* suppresses constitutively active transcription factors AP-1 and NF-κB, which resulted in the downregulation of expression of urokinase-type plasminogen activator (uPA) and its receptor uPAR in human breast and prostate cancer cells (8). The anticancer effect of *Ganoderma lucidum* was also demonstrated by the inhibition of adhesion, migration, and invasion of the highly metastatic breast cancer cells MDA-MB-231 (9).

The highly invasive potential of breast cancers was linked to the overexpression of epidermal growth factor receptors (EGFRs) (10), which control signaling through the activation of the phosphatidylinositol 3-kinase (PI3K)/NF-κB pathway responsible for the aberrant cell cycle progression of breast cancer cells (11). In addition, PI3K also activates serine/threonine protein kinase Akt (12), which can be specifically phosphorylated on Thr\(^{308}\) or Ser\(^{473}\) (13). Furthermore, activated Akt can subsequently regulate the NF-κB pathway (14,15), and NF-κB controls the expression of the cell cycle regulator cyclin D1, which is responsible for the transition from the G1 to the S phase during cell cycle progression (16). Finally, PI3K, Akt, and NF-κB are constitutively active in...
highly invasive human MDA-MB-231 breast cancer cells (17,18) and therefore they are suitable targets for cancer treatment (19). In the present study, we show that *Ganoderma lucidum* inhibits the growth of breast cancer cells by inducing cell cycle arrest at G0/G1 by suppressing NF-κB activity through the inhibition of Akt phosphorylation in MDA-MB-231 cells. We also report that *Ganoderma lucidum* suppresses the expression of cyclin D1 followed by the inhibition of cyclin-dependent protein kinase cdk4.

**Materials and Methods**

**Materials**

*Ganoderma lucidum* (Reishimax) was purchased from Pharmanex (Provo, UT). According to the manufacturer, this sample contains powdered extract (20:1) with spores and is standardized to 13.5% polysaccharides and 6% triterpenes. Stock solution was prepared by dissolving *Ganoderma lucidum* in sterile water at a concentration of 50 mg/ml and stored at 4°C.

**Cell Culture**

The human breast cells MCF-10A and breast cancer cells MDA-MB-231 were obtained from ATCC (Manassas, VA). MCF-10A cells were maintained in DMEM/F12 medium containing 5% horse serum (HS), insulin (10 μg/ml), epidermal growth factor (EGF; 20 ng/ml), cholera toxin (100 μg/ml), hydrocortisone (0.5 μg/ml), penicillin (50 U/ml), and streptomycin (50 U/ml). MDA-MB-231 cells were maintained in DMEM medium containing penicillin (50 U/ml), streptomycin (50 U/ml), and 10% fetal bovine serum (FBS). Media and supplements came from GIBCO BRL (Grand Island, NY). HS and FBS were obtained from Hyclone (Logan, UT).

**Cell Proliferation Assay**

Cell proliferation was determined by the tetrazolium salt method, according to the manufacturer's instructions (Promega, Madison, WI). Briefly, MCF-10A and MDA-MB-231 cells (5 x 10^5/well) were cultured in a 96-well plate and treated at indicated times with *Ganoderma lucidum* (0–1.0 mg/ml). At the end of the incubation period, the cells were harvested and absorption was determined with an ELISA plate reader at 570 nm. Data points represent mean ± standard deviation in one experiment repeated at least twice.

**Cell Cycle Analysis**

MDA-MB-231 cells (1 x 10^6) were seeded and after 24 h treated with *Ganoderma lucidum* (0.5 mg/ml) for the indicated period of time (0–48 h). After incubation, the cells were harvested by trypsinization, washed with Dulbecco's phosphate buffered saline (DPBS) containing 2% FBS, and resuspended in propidium iodine (50 μg/ml). Cell cycle analysis was performed on a FACStarPLUS flow cytometer (Becton-Dickinson, San Jose, CA), as previously described (20). Data are the mean ± standard deviation from 3 independent experiments.

**DNA Transfection and Chloramphenicol Acetyltransferase (CAT) Assay**

MDA-MB-231 cells were transfected with NF-κB-CAT reporter constructs and β-galactosidase expression vector pCH110, as previously described (8). Twenty-four hours after transfection, cells were treated with *Ganoderma lucidum* for an additional 24 h at 37°C, as indicated in the text. Cell lysates were prepared and CAT assays performed, as described (8). Data points represent the mean ± standard deviation of three independent transfection experiments.

**Western Blot Analysis**

MDA-MB-231 cells (1 x 10^7) were treated with *Ganoderma lucidum* (1.0 mg/ml) for 24, 48, 72, and 96 h. After incubation, cells were washed twice with ice-cold DPBS, lysed with 1 ml of ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EGTA, 1 mM EDTA, and protease inhibitor cocktail Complete™ [Boehringer Mannheim, Indianapolis, IN]) at 4°C for 30 min. The lysates were collected and cleared of nuclei by centrifugation for 10 min at 14,000 g. The equal amounts of proteins (20 μg/lane) were separated on 15% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). The protein expression was detected with the corresponding primary antibodies: anti-Akt, anti-phospho-Akt (Thr^308), anti-phospho-Akt (Ser^473); Cell Signaling, Beverly, MA), anti-cyclin D1, anti-Cdk4, and anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Protein expression was visualized using the ECL Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, UK).

**Results**

*Ganoderma lucidum* Inhibits Proliferation of Highly Invasive Human Breast Cancer Cells by Cell Cycle Arrest at G0/G1

We recently reported that *Ganoderma lucidum* inhibits the invasiveness of breast cancer cells by suppressing cell adhesion, cell migration, cell invasion, and colony formation (9). To demonstrate the effect of *Ganoderma lucidum* on cell growth, we treated MDA-MB-231 breast cancer cells with increasing concentrations of *Ganoderma lucidum* (0–7.0 mg/ml) for 24, 48, and 72 h, and cell proliferation was determined. As expected, *Ganoderma lucidum* suppressed the proliferation of MDA-MB-231 cells in a dose- and time-dependent manner (Fig. 1). To investigate the mechanism by which *Ganoderma lucidum* inhibits cell growth, we analyzed
Ganoderma lucidum inhibits proliferation of MDA-MB-231 cells. MDA-MB-3 cells were treated with 0, 0.25, 0.5, and 1.0 mg/ml of Ganoderma lucidum. Proliferation was assessed after 24, 48, and 72 h, as described in Materials and Methods. Each bar represents the mean ± standard deviation of 3 experiments.

Figure 2. Effect of Ganoderma lucidum on cell cycle distribution. MDA-MB-231 cells were treated with Ganoderma lucidum (0.5 mg/ml) for 0, 24, and 48 h, and the amount of cells at G0/G1, S, and G2/M phases were determined by flow cytometry, as described in Materials and Methods. Each bar represents the mean ± standard deviation of 3 experiments.

Ganoderma lucidum Supresses NF-kB Activity by Inhibiting Akt Kinase

We have previously demonstrated that purified spores or fruiting bodies of Ganoderma lucidum decrease NF-kB activity at the DNA-binding and at the transactivation levels in breast cancer cells (8). However, the amounts of biologically active components in our original samples of Ganoderma lucidum were not determined, which could result in a wide range of their potency to inhibit cancer cells (8,21). Thus, in the present study, we used Ganoderma lucidum in the form of powdered extract (20:1) with spores, which contains 13.5% polysaccharides and 6% triterpenes. In accordance with our previous data, Ganoderma lucidum inhibited constitutively active NF-kB in the reporter gene assay in MDA-MB-231 cells in a dose-response manner (Fig. 3).

Because Akt serine-threonine kinase controls the activity of NF-kB (14,15), we investigated whether the inhibitory effect of Ganoderma lucidum on NF-kB is mediated through the suppression of Akt in breast cancer cells. MDA-MB-231 cells were treated with Ganoderma lucidum (1.0 mg/ml) for 0, 24, 48, 72, and 96 h, and the expression of Akt was determined in whole cell extracts by Western blot analysis. As seen in Fig. 4A, Ganoderma lucidum inhibits the expression of Akt kinase in a time-response manner. However, the activity of Akt requires phosphorylation at Thr308 and Ser473 (22). Therefore, we investigated whether Ganoderma lucidum affects the phosphorylation of Akt. Although we did not observe significant inhibition of p-Akt- Thr308 (not shown), we found that Ganoderma lucidum markedly decreased phosphorylation of Akt at Ser473 (Fig. 4B). To determine whether the effect of Ganoderma lucidum on the activity of Akt is reversible, we treated MDA-MB-231 cells with Ganoderma lucidum (1.0 mg/ml), and after 24 and 48 h of incubation, culture media were replaced with media without Ganoderma lucidum, and the incubation continued for an additional 24 and 48 h. As shown in Fig. 4C, removal of Ganoderma lucidum restored the activity of Akt in MDA-MB-231 cells, as demonstrated by the
Figure 4. *Ganoderma lucidum* inhibits Akt in MDA-MB-231 cells. MDA-MB-231 cells were treated with *Ganoderma lucidum* (G1, 1.0 mg/ml) for the indicated times. Whole cell extracts were prepared and subjected to Western blot analysis with A: anti-Akt and B: anti-p-Akt-Ser^{473} antibodies, as described in Materials and Methods. C: MDA-MB-231 cells were untreated for 24 h (1), and 48 h (2), or treated with G1 for 24 h (3), and 48 h (4), or treated with G1 for 24 h followed by the incubation with fresh media for an additional 24 h (5) and 48 h (6), or treated with G1 for 48 h followed by the incubation with fresh media for an additional 24 h (7) and 48 h (8). Western blot analysis with anti-p-Akt-Ser^{473} antibodies was performed as described previously. The equivalent amount of protein was verified by reprobing the blots with anti-β-actin antibody. The results are representative of 3 separate experiments.

Phosphorylation of Akt at Ser^{473}. Thus, *Ganoderma lucidum* inhibits constitutively active NF-κB by suppressing Akt kinase activity, and the effect of *Ganoderma lucidum* on Akt activity is reversible.

**Ganoderma lucidum** Downregulates the Expression of Cyclin D1 and cdk4

Because Akt kinase controls the activation of NF-κB (23) and because NF-κB regulates the expression of cyclin D1 (16), we hypothesized that cell cycle arrest at G0/G1 by *Ganoderma lucidum* is the result of downregulation of the expression of cyclin D1. The cell extracts from MDA-MB-231 cells treated with *Ganoderma lucidum* were subjected to Western blot analysis with anti-cyclin D1 antibody. As shown in Fig. 5A, *Ganoderma lucidum* markedly decreased the expression of cyclin D1 in a time-response manner. Because cyclin D1 regulates the activity of cyclin-dependent protein kinase cdk4, which controls the G1 cell cycle checkpoint (24), we investigated the effect of *Ganoderma lucidum* on cdk4 kinase. As expected, *Ganoderma lucidum* also inhibits expression of cdk4 (Fig.
Figure 5. *Ganoderma lucidum* downregulates the expression of cyclin D1 and cdk4 in MDA-MB-231 cells. MDA-MB-231 cells were treated with *Ganoderma lucidum* (1.0 mg/ml) for the indicated times. Whole cell extracts were subjected to Western blot analysis with A: anti-cyclin D1 and B: anti-cdk4 antibodies. The equivalent amount of protein was verified by reprobing the blots with anti-β-actin antibody. The results are representative of 3 separate experiments.
The enzymatic activity of Akt3 was also significantly downregulated in invasive and metastatic cancers. Our data demonstrate that Akt is a suitable target for inhibiting highly invasive cancer cells, further confirming the role of Akt as an oncogene responsible for the survival and growth of cancer cells (30). Therefore, Akt is a suitable target for inhibiting highly invasive and metastatic cancers. Our data demonstrate that Ganoderma lucidum downregulates the expression of Akt and suppresses the activity of Akt by inhibiting phosphorylation of Akt at Ser473, which is the regulatory phosphorylation site responsible for the activity of Akt. Although Akt activity can be suppressed by specific synthetic inhibitors of PI3K such as wortmannin and LY294002, here we show the inhibition of Akt with the mushroom extract from Ganoderma lucidum. Interestingly, other natural products, such as epigallocatechin-3-gallate (EGCG), a major bio-logically active component of green tea, and genistein, an isoflavonoid from soy beans, inhibitedAkt in MDA-MB-231 breast cancer cells (31, 32). However, EGCG inhibited TGF-α-dependent phosphorylation of Akt at Ser473, whereas the same treatment did not affect the levels of Akt expression (31). On the other hand, genistein inhibited both the kinase activity of Akt as well as the expression of total Akt and phosphorylated Akt at Ser473 (32). Considering all of these findings, we can conclude that Ganoderma lucidum downregulates the expression of constitutively active Akt and inhibits phosphorylation of Akt at Ser473.

In the present study, we also demonstrated that Ganoderma lucidum suppresses the activity of NF-κB in MDA-MB-231 cells. The suppression of NF-κB activity by Ganoderma lucidum is probably modulated through the inhibition of Akt kinase, which controls the activity of NF-κB by phosphorylation of IκB kinase (IKK) and liberation of NF-κB from IκB by degradation of IκB (14, 15). Our data are consistent with studies by Masuda et al., which demonstrate that the suppression of Akt by EGCG results in the inhibition of constitutively active and TNF-α-induced NF-κB in a reporter gene assay (31). Furthermore, Gong et al. show that genistein, which inhibits Akt, also inhibits the DNA-binding activity of NF-κB in unstimulated or EGF-stimulated MDA-MB-231 cells (32). Alternatively, the effect of Ganoderma lucidum on the activity of NF-κB can be caused by the inhibition of PKC, and we have also recently demonstrated that the PKC inhibitor bisindolylmaleimide I suppresses NF-κB activity in MDA-MB-231 cells (33). Finally, recent studies demonstrate that polysaccharides isolated from Ganoderma lucidum inhibit alloxan-induced activation of NF-κB in pancreas (34) and that the herbal mixture PC-SPES, which also contains Ganoderma lucidum, suppresses lipopolysaccharide-induced NF-κB in macrophages (35).

As we demonstrated previously, Ganoderma lucidum inhibits cell proliferation and induces cell cycle arrest at G0/G1. As we expected, this effect is a result of the inhibition of cyclin D1, the expression of which is controlled by NF-κB (16). Subsequently, inhibition of cyclin D1 suppressed cdk4 kinase, which resulted in cell cycle arrest at G0/G1 and the inhibition of proliferation. In addition to inducing cell cycle arrest, extended exposure of Ganoderma lucidum also induced apoptosis in MDA-MB-231 cells (not shown). Experiments are in progress to characterize the mechanism(s) by which Ganoderma lucidum induces apoptosis in breast cancer cells. A recent study suggests that EGF-induced NF-κB activation is a major pathway for the proliferation of MDA-MB-231 cells, and the inhibition of NF-κB suppressed regulatory proteins of G1/S cell cycle progression, such as
cyclin D1 and pRb (11). In addition to controlling cell proliferation, NF-κB also controls the expression of proteins responsible for metastasis and angiogenesis of cancers (19); we have recently demonstrated that *Ganoderma lucidum* inhibits NF-κB-dependent expression of uPA and uPAR, which resulted in the inhibition of the metastatic behavior of highly invasive breast cancer cells (8,9).

Cell cycle arrest at G0/G1 phase and inhibition of cyclin D1 by the alcohol extract of *Ganoderma lucidum* was also reported by Hu et al. (36). However, this effect was observed in breast cancer MCF-7 cells (36), cancer cells that do not contain constitutively active NF-κB and are not as invasive as the highly metastatic MDA-MB-231 cells (17). We have also found that *Ganoderma lucidum* inhibits proliferation of the normal breast cells MCF10A (not shown), cells that also do not have constitutively active NF-κB, nor do they overexpress cyclin D1 (37,38), suggesting that *Ganoderma lucidum* inhibits cell proliferation by multiple mechanisms. Furthermore, ethanol extracts of *Ganoderma lucidum* inhibited growth and induced cell cycle arrest at the G0/G1 phase of human cervical cancer HeLa cells (39). Alternatively, Lin et al. (40) recently described the inhibition of growth and G2 cell cycle arrest by *Ganoderma lucidum* in human hepatoma cells, which were linked to the downregulation of expression of cyclin B, and we found that *Ganoderma lucidum* arrested PC-3 prostate cancer cells at the G2/M phase independently of the inhibition of NF-κB (41). All of these findings together suggest that *Ganoderma lucidum* can inhibit the proliferation of normal and cancer cells by specific mechanism(s), which are different in various cell lines.

In summary, our data demonstrate that *Ganoderma lucidum* inhibited the growth of human breast cancer cells by cell cycle arrest at the G0/G1 phase. The biological effects of *Ganoderma lucidum* on MDA-MB-231 cells are mediated by the inhibition of phosphorylation and downregulation of expression of Akt kinase, which results in the suppression of NF-κB activity following the downregulation of cyclin D1 and cdk4 expression. The study presented demonstrates how the edible mushroom *Ganoderma lucidum* inhibits the growth of highly invasive and metastatic breast cancer cells on the molecular level and could validate its possible use for the prevention and/or treatment of breast cancer.

**Acknowledgments and Notes**

We thank Dr. Karen Spear for editing the manuscript. This work was supported by a grant from the Showalter Foundation to D. Silva. Address correspondence to D. Silva, Cancer Research Laboratory, Methodist Research Institute, 1800 N Capitol Ave, E504, Indianapolis, IN 46202. Phone: (317) 962–5371. FAX: (317) 962–9369. E-mail: dsliva@clarian.org.

Submitted 11 February 2004; accepted in final form 15 June 2004.

**References**


33. Sliva D, English D, Lyons D, and Lloyd Jr FP: Protein kinase C induces motility of breast cancers by upregulating secretion of urokinase-type plasminogen activator (uPA) through the activation of AP-1 and NF-κB. *Biochem Biophys Res Commun* 290, 552–557, 2002.


