The Mushroom Extract Schizophyllan Reduces Cellular Proliferation and Induces G2/M Arrest in MCF-7 Human Breast Cancer Cells

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Abstract: Breast cancer is the most common cancer in women worldwide. It is also the principle cause of death from cancer among women globally**.** Several lines of evidence suggest that dietary mushrooms may decrease breast cancer incidence. Schizohyllan (SCH) is a polysaccharide isolated from the medicinal mushroom *S. commune* and has potential anticancer effects. In a recent study we have demonstrated that SCH alone or in combination with tamoxifen (TAM) reduced the incidence of 7, 12 Dimethylbenz(α)anthracene (DMBA)-induced mammary carcinomas in mice through inhibition of cellular proliferation. The goal of the present work was to study the molecular mechanism through which SCH inhibits cell proliferation using the estrogen receptor positive human breast cancer cells (MCF-7) in vitro. MCF-7 cells were treated with different concentrations of SCH, and the following parameters were studied: cell growth, apoptosis, cell cycle kinetics using flow cytometry and expression of cell cycle regulatory proteins by Western blot. It was found that 1500 µg/ml of SCH reduced cell viability and this was not due to cell death by apoptosis but due to G2/M cell cycle phase arrest. Furthermore, the molecular mechanism underlying the G2/M phase arrest involved an increased phosphorylation at the inhibitory tyrosine 15 site of CDK1 associated with accumulation of p53. Taken together, this is the first study to show direct anticancer effects of SCH on human breast cancer cells in culture. The therapeutic implications of SCH in human breast cancer warrant further investigation.

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1. Introduction

Breast cancer is the most common cancer in women worldwide. It is also the principle cause of death from cancer among women globally (Parkin, 2008). Treatment options for breast cancer patients include surgery, radiation therapy, chemotherapy, and targeting therapies. However, most drugs for chemoprevention have deleterious side effects. It has been reported that long-term administration of tamoxifen (TAM), a widely used antiestrogenic drug for chemotherapy and for the chemoprevention of breast cancer (Jordan, 2003), results in serious adverse effects including endometrial cancer (Killackey *et al.*, 1985; Bernstein *et al.*, 1999). Mushroom species or their constituent bioactive agents have been tested against several major forms of human cancer including stomach, colon, lung and liver and have been shown to be protective in numerous experimental models (Kidd, 2000). Several lines of evidence suggest that dietary mushrooms may decrease breast cancer incidence. In a case-control study of Chinese women with breast cancer, dietary intake of 10 g of fresh mushrooms or 4 g of dried mushroom powder significantly decreased breast cancer in pre- and postmenopausal women (Zhang *et al.*, 2009). Furthermore, (Kodama *et al.*, 2002) reported that 69% of breast cancer patients consuming whole maitake mushroom powder showed significant cancer regression and marked symptom improvement. In cancer patients the maitake D-fraction, an antitumor polysaccharide fraction, hindered metastatic progression, reduced the expression of tumor markers and increased natural killer cell activity in all patients (Kodama *et al.*, 2003). Schizohyllan (SCH) is a polysaccharide isolated from the inedible mushroom *S. commune* (Mwt ~ 450 kD). It inhibits solid Sarcoma 180 tumor when injected by intraperitoneal or intravenous route (Komatsu *et al.*, 1969). In cervical cancer, SCH prolonged survival and time to recurrence for stage II but not stage III cases (Okamura *et al.*, 1989; Miyazaki *et al.*, 1995) and showed added effectiveness when injected directly into the tumor mass (Nakano *et al.*, 1996). In a randomized trial SCH, also known as SPG, combined with conventional chemotherapy improved the long term survival rate of patients with ovarian cancer (Inoue *et al.*, 1993). In a recent study (Aleem et al., 2011) have demonstrated that SCH alone or in combination with TAM reduced the incidence of 7, 12 Dimethylbenz(α)anthracene (DMBA)-induced mammary carcinomas in mice. The DMBA-induced mammary carcinomas were estrogen receptor-positive

(ER+ve). SCH and TAM equally decreased the proliferating cell nuclear antigen (PCNA) labeling index, a marker for proliferating cells, relative to DMBA in mammary tumors. These mice developed hepatocellular carcinoma (HCC) as well and SCH but not TAM reduced the development of HCC, decreased PCNA labeling index in HCC as well as increased the levels of caspase-3 expression. It is noteworthy to mention that SCH did not have any adverse side effects on normal mammary gland or liver (Aleem et al., 2011). The results of the in vivo study prompted the further investigation of the molecular mechanisms of anticancer effects of SCH in vitro in an ER+ve breast cancer cell line (MCF-7). In the present study SCH reduced cell proliferation, and induced cell cycle arrest at the G2/M phase, however it did not induce apoptosis. The G2/M arrest in the present work was caused by an increased tyrosine 15 phosphorylation of CDK1 and gradual accumulation of p53.

2 Materials and Methods

Cell culture

The human breast cancer cell line MCF-7 (ATCC, USA) was maintained in culture in DMEM containing glucose, sodium puyruvate and glutamine (Gibco, UK) supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 units/ml), and streptomycin (50 µg/ml) (Gibco, UK). Cells were maintained at 37 ℃ in a humidified atmosphere containing 5% CO_2 and treated with different concentrations of SCH (CPN spol. s.r.o, Czech Republic) dissolved in sterile water.

Viability assay

Cell viability was measured using alamar blue® viability assay (Ahmed *et al.*, 1994) according to manufacturer's protocol. Briefly, cells were seeded at a density of 5000 cells per one well of 96-well plates in triplicates and allowed to grow for 24 hrs. Different concentrations of SCH were added and cells incubated for four days. Four hours before analysis 10% of alamar blue®solution diluted in culture medium was added and cells were incubated at 37 ℃. Fluorescence was measured at 550/590 nm using a microplate reader.

Cell Cycle and flow cytometry

Cells were seeded in 10 cm dishes, allowed to grow until subconfluency then treated with different concentrations of SCH for 24 hrs**.** Cells were then trypsinized, collected by centrifugation and washed twice with PBS then incubated for 10 min at 37^{o}C in (0.34 mM Trisodium citrate.2 H20, 0.1% NP-40, 1.5 mM spermine tetrahydrochloride and 0.5 mM Tris (hydroxymethy1)-aminomethane containing trypsin) followed by another 10 min incubation at 37^{o}C in an equal volume of the same buffer, however, trypsin was substituted with a trypsin inhibitor. An equal volume of propidium iodide (PI) was added and cells were incubated for at least 15 min on ice in the dark. The samples were then finally analysed with a FACS Calibur (Becton Dickinson), and the data were processed with the Cell Quest software (Becton Dickinson).

Apoptosis assay

Cells were seeded in 96-well plates, treated with different concentrations of SCH for 48 h then processed for apoptosis analysis using the M30 Apoptosense®Elisa kit (Peviva Ab, Sweden) according to manufacturer's protocol.

Preparation of lysates and Western blot

Cells were lysed in modified RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% sodium deoxycholate). Proteases inhibitor cocktail (Roche, Germany) and phosphatase inhibitors (Sigma-aldrich, Germany) were freshly added. Lysates were centrifuged for 30 min at 16,100 x*g* at 4 ℃, and supernatants were frozen at -80 ℃ until use. Protein concentrations were determined using the BCA protein assay (Pierce Biotechnology, Rockford, USA) according to manufacturer's protocol and concentrations measured in a microplate reader. Lysates (20 µg total protein per lane) were resolved on 12 % Bis-Tris SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes (Hybond, Amersham, UK), blocked with 5% milk in a Tris-Buffered Saline and Tween 20 (TBST) [19.97 mM Tris base, 135 mM NaCl, 0.1% Tween 20] or 5% Bovine Serum Albumin in TBST, and blotted with the following primary antibodies: mouse anti-p53 (Becton Dickenson, USA), rabbit anti-Cdk1 (Calbiochem), rabbit anti-phospho-Y15 Cdk1 (Cell Signaling Technology, USA), rabbit anti-cyclin D (Cell Signaling Technology, USA) and anti- -tubulin (Sigma-aldrich, Germany). Membranes were routinely washed using TBST and incubated with anti-rabbit- or anti-mouse-IgG horseshoe peroxidase (HRP)-conjugated secondary antibody (Amersham, UK) and protein bands detected with the ECL chemiluminescence system (Amersham, UK). The bands were scanned and analysed by densitometry using ImageJ 1.44p software (Wayne Rasband, NIH, USA).

Statistics

Data were analysed for mean and standard deviation and significance was determined using student's t test. A p value ≤ 0.05 was considered significant.

3. Results

SCH reduces viability of MCF-7 cells

In the present study MCF-7 cells were treated with different concentrations of SCH (250, 500, 1000, and 1500 µg/ml) and cell viability was measured. The 250 and 500 µg/ml SCH concentrations did not have an effect on viability of MCF-7 cells (Fig.1). After 48 hrs of treatment the surviving fraction of cells treated with 1000 and 1500 was 75% and 50%, respectively (Fig. 1). There were no surviving cells after treatment with 2000 µg/ml (data not shown).

SCH does not induce apoptosis

In order to investigate why SCH reduced viability of breast cancer cells MCF-7 cells were treated with different concentrations of SCH for 48 hrs and measured apoptosis using M30 Apoptosense®Elisa assay (Fig. 2). Although SCH did induce some apoptotic death at high concentrations, however, the values were not statistically significant. This result was also confirmed by flow cytometry (Fig. 3E, the sub-G1 fraction). The values in Fig. 2 are expressed as fold change relative to untreated cells. The highest SCH concentration (1500 µg/ml) induced only a 1.6 fold increase in apoptotic cell death in comparison to untreated cells (Fig. 2).

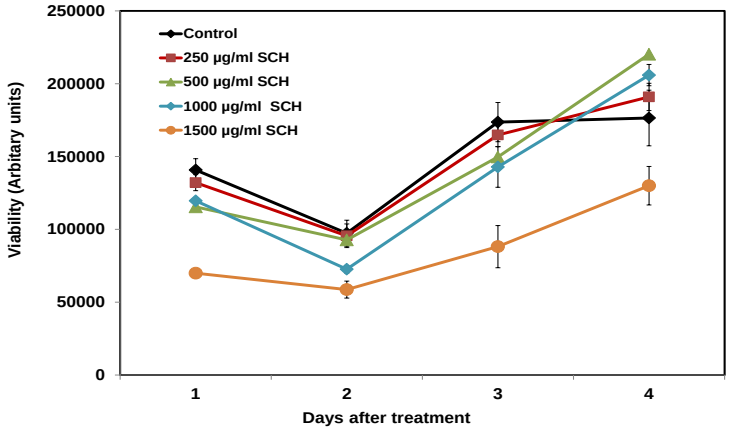

Figure 1. Schizophyllan reduces viability of MCF-7 cells in a dose-dependent manner. Cells were seeded in 96-well plates, treated with different concentrations of schizophyllan and their viability was monitored daily for 4 days. Viability was measured using alamar blue® viability assay.

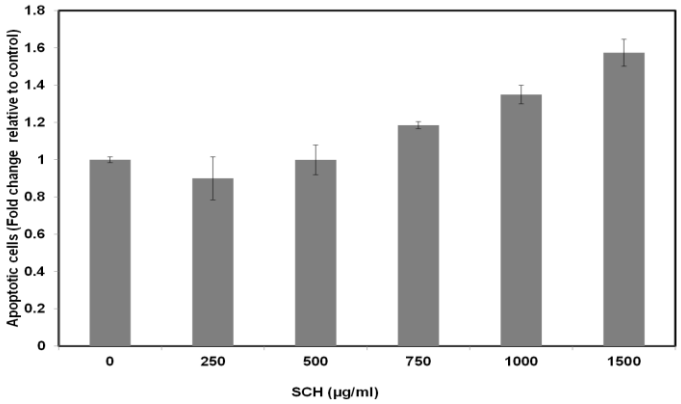

Figure 2. Schizophyllan does not induce apoptosis in MCF-7 cells. Cells were seeded in 96-well plates, treated with different concentrations of schizophyllan and apoptosis was scored after 48 hrs using M30 Apoptosense®Elisa assay.

SCH induces G2/M arrest

Analysis of the cell cycle profile by flow cytometry in control and SCH-treated MCF-7 cells revealed that SCH induced a gradual dose-dependent accumulation of cells at the G2/M phase, which was accompanied with a concomitant decrease in the population of cells in G1 phase (Fig. 3). While the 500 µg/ml concentration of SCH caused a 2.4 fold increase in the percentage of cells at G2/M phase, the 1000 and 1500 µg/ml concentrations induced a 3.6 and 3.7 fold increase in comparison to untreated cells, respectively (Fig. 3 A, B, C, D, E).

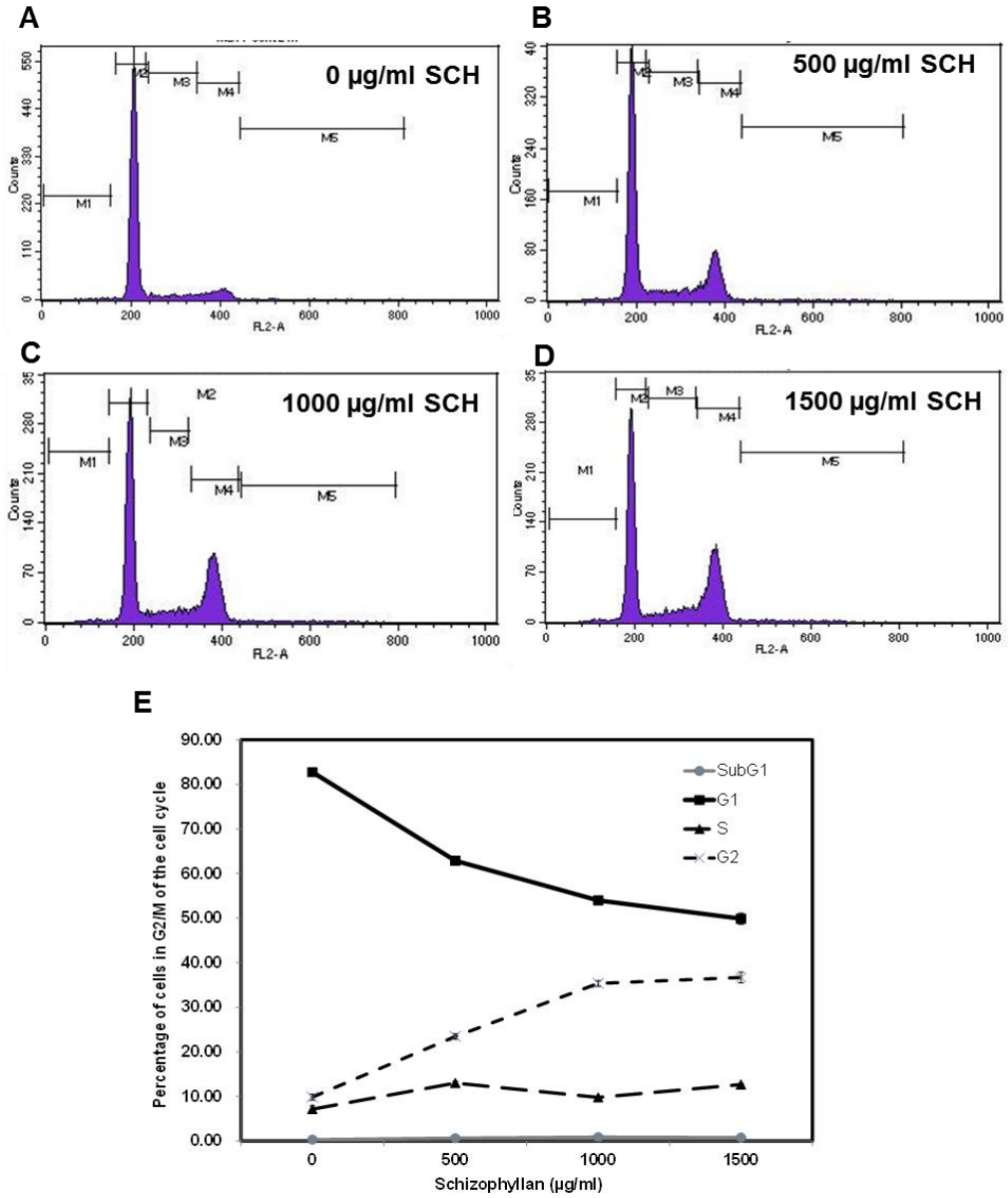

Figure 3. Schizophyllan induces G2/M arrest in MCF-7 cells. Cells were seeded in 10 cm dishes, treated with 0 µg/ml (A), 500 µg/ml (B), 1000 µg/ml (C) and 1500 µg/ml schizophyllan (D) for 24 hrs and the cell cycle profiles were then measured using propidium iodide staining and flow cytometry. Data were analysed using Cell Quest software.

SCH increases tyrosine 15 phosphorylation of CDK1 and induces p53 accumulation

To investigate the mechanisms underlying the G2/M cell cycle arrest the protein levels of CDK1, the kinase regulating G2/M phase transition, and its phosphorylation status, as well as p53, which is one of the major proteins regulating G2 arrest were studied. SCH did not affect the levels of CDK1 protein but it significantly increased the tyrosine15 (Y15) phosphorylation (Fig. 4A). Densitometric analysis revealed a 4.5 fold increase in the Y15 phosphorylation by 1500 µg/ml SCH compared to control cells (Fig. 4B). Furthermore, SCH induced p53 accumulation in a dose-dependent dose. The highest concentration (1500 µg/ml) of SCH induced a 2.3 fold increase in p53 levels relative to control (Fig. 4C). In addition, SCH did not alter the levels of Cyclin D1, a major regulatory protein during G1 phase progression. The membrane was stripped and incubated with -tubulin as loading control (Fig. 4A).

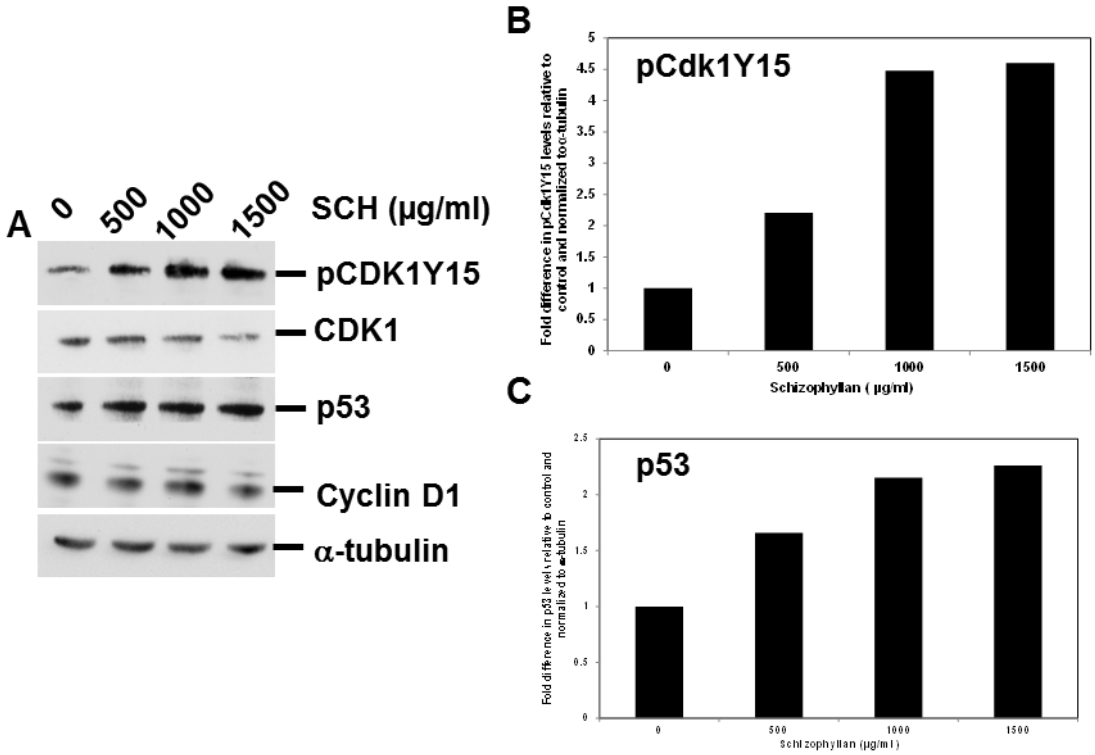

Figure 4. Schizophyllan increased the tyrosine 15 phosphorylation of CDK1 and induced accumulation of p53 in MCF-7 cells. Lysates were prepared from MCF-7 cells treated with 0 µg/ml, 500 µg/ml, 1000 µg/ml and 1500 µg/ml schizophyllan for 24 hrs, run on 12% SDS-PAGE, and analysed by Western blot (A). The intensities of the pCDK1Y15 and p53 protein bands normalized to -tubulin were studied by densitometry, and the values plotted in (B) and (C), respectively.

4. Discussion

Medicinal mushrooms have been shown to have promising antitumor, immune modulating, antiviral and antiparasitic effects (Wasser and Weis, 1999). In a recent study (Aleem et al., 2011) demonstrated for the first time that schizophyllan (SCH), a -D-glucan isolated from *S. commune* inhibited mammary and hepatic carcinomas induced in mice by DMBA through reduction of cell proliferation in both mammary and liver tumors and induction of apoptosis in liver but not in mammary tumors. The goal of the present study was to investigate the molecular mechanisms underlying the inhibition of cell proliferation using human breast cancer cells in vitro.

In the present work, treatment of the human breast cancer cell line MCF-7 with different concentrations of SCH resulted in significant reduction of cell growth at the 1500 µg/ml concentration. It has been previously reported that mushroom polysaccharides primarily exert their antitumor activity via activation of the immune response of the host organism (Kim *et al.*, 2006), which may imply that they do not have a direct effect on tumor cells. However, the present study and previous reports have demonstrated direct effects of mushroom extracts on cell growth, cell cycle and apoptosis of several human cancer cell lines in vitro.

This is the first study to report a direct effect of the inedible mushroom schizophyllan on breast cancer cells in vitro, and is consistent with a previous study showing that extracts from five different edible mushrooms significantly suppressed cellular proliferation in MCF-7 cells (Martin and Brophy, 2010). Furthermore, extracts from PORT, shiitake and OYS mushrooms suppressed proliferation of both the ER-positive MCF-7 and the ER-negative MDA-MB-231 human breast cancer cells, without affecting proliferation of normal cells

(Jedinak and Sliva, 2008). In agreement with these results, extracts from *Inonotus obliquus* showed also antiproliferative effect on HT-29 cancer cells (Lee *et al.*, 2009), as well as on melanoma B16-F10 and HepG2 liver cancer cells (Youn *et al.*, 2008; Youn *et al.*, 2009). In the present study, it was found that the reduction in cell proliferation of MCF-7 cells was not due to cell death by apoptosis but due to G2/M cell cycle phase arrest. This result supports our recent in vivo study in which we demonstrated that SCH markedly reduced cell proliferation in DMBA-induced mammary carcinomas in mice without affecting the levels of caspase-3 expression, a central protein in the execution of apoptosis (Aleem et al., 2011). Similarly, (Martin and Brophy, 2010) found only one mushroom species out of five (Maitake) that induced apoptosis in addition to inhibition of cell growth in MCF-7 cells. The results from the present study are also consistent with previous studies that demonstrated that extracts from the mushroom *Agaricus blazei* induced an arrest at the G2/M phase of the cell cycle in human gastric epithelial AGS cells (Jin *et al.*, 2006). Furthermore, the current study demonstrated that the molecular mechanism underlying the G2/M phase arrest by SCH is due to increased phosphorylation at the tyrosine 15 inhibitory site of CDK1 coupled with an accumulation of p53. It is known that CDK1 is the central regulator of mitosis and it has to bind to cyclin B in mitosis to be activated (Desai *et al.*, 1992). CDK1 bound to cyclin B is phosphorylated on residue T161 by CDK- activating kinase (CAK) to stabilize the cyclin B-CDK1 interaction and to induce the conformational rearrangements needed for kinase activity (Russo *et al.*, 1996; Larochelle *et al.*, 2007). However, the WEE1 and MYT1 kinases rapidly inactivate CDK1 by phosphorylating residues T14 and Y15, thereby blocking ATP binding and hydrolysis. Consequently, at low levels of cyclin B, CDK1 is inactive (Solomon *et al.*, 1990). Once cyclin B concentrations exceed a threshold, CDK1 is activated. Cyclin B-CDK1 phosphorylates and activates the CDC25 phosphatase, allowing CDC25 to remove the inhibitory T14 and Y15 phosphorylations on CDK1. Cyclin B-CDK1 is also a negative regulator of both MYT1 and WEE1, as these two kinases are inactivated upon cyclin B-CDK1 phosphorylation (Watanabe *et al.*, 1995; Okamoto and Sagata, 2007). Therefore, in the present study the increased Y15 phosphorylation induced by SCH deactivated CDK1 and this may be a potential mechanism leading to an accumulation of cells in the G2/M cell cycle phase. Furthermore, SCH induced the accumulation of p53. p53 protects mammals from neoplasia by inducing apoptosis, DNA repair and cell cycle arrest in response to a variety of stresses (Taylor and Stark, 2001). P53 regulates the G2/M transition through several mechanisms. Part of the mechanism by which p53 blocks cells at the G2 checkpoint involves inhibition of CDK1. CDK1 is inhibited simultaneously by three transcriptional targets of p53, Gadd45, p2l, and l4-3-3a (Taylor and Stark, 2001). Repression of the cyclin B1 gene by p53 also contributes to blocking entry into mitosis. p53 also represses the *cdk1* gene, to help ensure that cells do not escape the initial block. Repression of the topoisomerase II gene by p53 helps to block entry into mitosis and strengthens the G2 arrest (Taylor and Stark, 2001). Consistent with the present results (Zhou *et al.*, 2011) reported a G2/M phase arrest induced by *Ganoderma lucidum* (mushroom) and this arrest was associated with an accumulation of p53 in ovarian cancer cells.

Taken together, results from the present study coupled with recent results in vivo (Aleem et al., 2011) provide a strong line of evidence that schizophyllan exerts multiple antitumor effects on human and mouse breast cancer cells. It inhibits cell proliferation and induces cell cycle phase arrest associated with the induction of p53, and therefore, its therapeutic implications in breast cancer warrant further investigation.

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