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Article

Lp16-PSP, a member of YjgF/YER057c/UK114 Protein Family Induces Apoptosis and p21$^{WAF1/CIP1}$ mediated G_1 Cell Cycle Arrest in Human Acute Promyelocytic Leukemia (APL) HL-60 Cells

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Abstract:

Lp16-PSP from *Lentinula edodes* strain C_{91-3} has been reported previously in our laboratory to have selective cytotoxic activity against a panel of human cell lines. Herein, we have used several parameters in order to characterize the Lp16-PSP-induced cell death using HL-60 as model cancer. The results of phase contrast microscopy, nuclear examination, DNA fragmentation detection and flow cytometry revealed that high doses of Lp16-PSP resulted in the induction of apoptosis in HL-60 cells. The colorimetric assay showed the activation of caspase-8, -9 and -3 cascade highlighting the involvement of Fas/FasL-related pathway. Whereas, western blot revealed the cleavage of caspase-3, increased expression of Bax, the release of cytochrome c and decreased expression of Bcl-2 in a dose-dependent manner, suggesting the intrinsic pathway might be involved in Lp16-PSP-induced apoptosis either. Low doses of Lp16-PSP resulted in the anchorage-independent growth inhibition, induction of G_1 phase arrest accompanied by the increased expression of p21$^{WAF1/CIP1}$ along with the decreased expression of cyclin D, E, and cdk6. Our findings suggest that induction of apoptosis and p21$^{WAF1/CIP1}$ mediated G_1 arrest might be one of the mechanisms of the action of Lp16-PSP, however, further investigations on multiple leukemia cell lines and *in vivo* models are of ultimate need.

Keywords: *Lentinula edodes*; Lp16-PSP; Acute Promyeloid Leukemia; Extrinsic and Intrinsic Apoptotic Pathway; G_1 Phase Cell Cycle Arrest

1. Introduction:

In the United States, approximately after every three minutes, a person is diagnosed with hematological cancer [1]. Leukemia is one of the types of blood cancer that usually initiates in blood-forming organs including bone marrow, followed by the increment in abnormal leukocytes numbers. On the basis of pathological features, leukemia can be classified as acute and chronic leukemia. Where acute leukemia can be acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL), and on the other hand chronic leukemia can be chronic myeloid leukemia (CML) or chronic lymphocytic leukemia (CLL) [2]. As far as acute myeloid leukemia (AML) is concerned it is characterized by the malignant hematopoietic progenitor cells (HPCs) accumulation that have impaired differentiation program [3]. In the United States alone, around 21,380 new cases of acute myeloid leukemia (AML) are expected to occur in the year 2017 with an overall five-year survival rate of 26.9% [1].

Acute promyeloid leukemia (APL) is the clear subtype of AML, that is caused by the leukocyte differentiation arrest at the promyelocyte stage and was considered as fatal before the discovery of all-trans retinoic acid (ATRA) the derivative of vitamin A [4]. In the treatment of APL patients, risk stratification is considered to be crucial, and so less intensive regimens are adapted for treating the patients at low risk having white blood cell count (WBC) of ≤10,000/ul, in comparison with patients presenting high-risk disease (WBC 410,000/µl). Initially, APL patients were defined as low risk for relapse (WBC ≤10 000/µl and platelet count 440,000/µl), intermediate risk (WBC ≤10000/µl and platelet count ≤ 40k), and high risk (WBC 410,000/µl) on the basis of cell count [5]. But because, low- and intermediate-risk patients have common outcomes, and so they were collectively considered as a low-risk disease. As far as therapy for newly diagnosed APL patients is concerned, in the last two decades, it has excogitated from an all-trans retinoic acid (ATRA) + chemotherapy to the arsenic trioxide (ATO) addition followed by the chemotherapy omission in low-risk patients [6].

Natural products, from higher plants, fungi, and microorganisms have a long history of being used as therapeutic agents for the treatment of several diseases. The compounds from natural resources have proven their therapeutic role in unmodified form as well as in contributing a variety of derivatives called secondary metabolites [7]. Concerning the role of natural products in the treatment of leukemia, L-asparaginase, daunorubicin, and the anthracyclines are well-known for their anti-leukemia activities [8]. Sucessfulment of the treatment not just depends on the category, but also on the genetic factors associated with each disease. Chemotherapy, radiation therapy, antibiotics usage, transfusion and transplantation of blood and bone marrow are some of the strategies used in combination to treat leukemia patients respectively. Although these strategies resulted in prolonged survival, however, some of these treatments are difficult to handle [9]. In order to make advances in our journey toward curative therapy, there is a continuous need of identifying the novel protective mechanisms and pathways responsible for the survival of tumor, as we can not only rely on the presently available arsenals.

In general, if we look into the strategies for combating cancer, in the flow of genetic information, the expression of the gene (s) in the cancer cells can be controlled at different levels, but the drug that targets DNA have the disadvantage of being mutagenic. In contrast, the agents that target RNA are significantly effective without having genotoxic effects [10]. Current strategies to combat cancer are not always effective,

because of the development of resistance, severe side effects, and diversity of the target in the cancer cells. The cancer biologists are targeting the RNA pool of cancer cells as alternative strategies for combating cancer, as it differs significantly from the normal cells highlighting the additive advantage of selective toxicity. In this regard, many of the characterized ribonucleases from various organisms have displayed antitumor activities [11-14] and efforts to increase such arsenals are still ongoing, especially with the natural resources.

Keeping in mind this alternative strategy to combat cancer and based on the bioinformatics analysis, our laboratory has demonstrated that Lp16-PSP from one of the edible mushroom *Lentinula edodes* $C_{91\text{-}3}$ is an endoribonuclease L-PSP and is a member of highly conserved YjgF/YER057c/UK114 protein family. Furthermore, we demonstrated the selective anticancer activity of Lp16-PSP against a panel of human cell lines and acute promyeloid leukemia HL-60 cell line was identified as the most sensitive cell line with the IC_{50} value of 74.4 ± 1.07 μg/ml after 48 h treatment [15]. Therefore, the objective of this study is to use human acute promyeloid leukemia (HL-60 cells) as model cancer to further investigate the potential molecular mechanism of the action of Lp16-PSP. We thus investigated several parameters such as DNA fragmentation, mitochondrial membrane potential, *Bax*/*Bcl-2* expression, activation of caspases, and cell cycle distribution, in HL-60 cells as *in vitro* model system. In this study, we observed that Lp16-PSP resulted in the increased expression of *FasL*, together with the loss of mitochondrial membrane potential and the release of cytochrome c, indicating that extrinsic and intrinsic pathway might be involved in the induction of apoptosis. Furthermore, Lp16-PSP also resulted in the anchorage-independent growth inhibition and $p21^{WAF1/CIP1}$ mediated G_1 cell cycle arrest in HL-60 cells.

2. Results:

2.1 Lp16-PSP causes cytotoxicity and suppresses the anchorage-independent colony formation of HL-60 cells: After treatment of the HL-60 cells with indicated concentrations of Lp16-PSP, phase contrast images were taken. As shown in Figure. 1A treated HL-60 in comparison with the untreated group, showed an obvious change in morphology, cell volume and size. The cells in the treated group are smaller in size and also showing the cellular bleeding. All these signs indicated that the HL-60 cells are going through the process of apoptosis or are dead. These findings highlighted the implication of Lp16-PSP as a potential anticancer agent against acute promyeloid leukemia (APL).

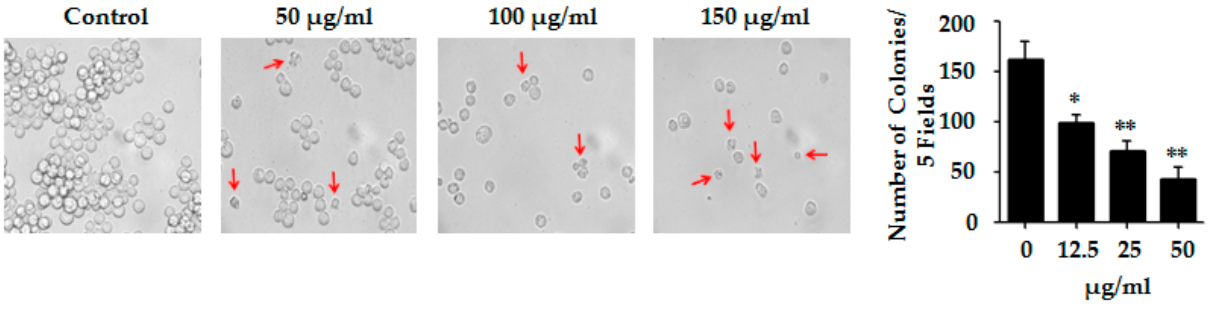
Metastatic malignant cells have the ability to resist the detachment-induced death, which helps them to grow and survive during the period of their dissemination [16]. The HL-60 has been reported previously to have the outstanding property of proliferating and forming sizable colonies in soft agar from a single cell [17]. In order to determine whether Lp16-PSP can affect the HL-60 colony formation, HL-60 cells were mixed with soft agar and cultured till the development of visible colonies. Colonies so formed in the agar were counted carefully. Our results indicated the significant dose-dependent effect of Lp16-PSP on HL-60 colony formation in semisolid agar. These results also indicate that Lp16-PSP treatment resulted in the suppression of HL-60 colony formation without any apparent cytotoxicity at the concentrations of Lp16-PSP used (Fig. 1B).

2.2 Lp16-PSP-Induced Apoptosis in acute promyeloid leukemia (APL) HL-60 cells: Apoptosis or programmed cell death is characterized by certain typical features i.e. shrinkage of the cell, condensation of nuclear chromatin, cleavage of chromosomes, bleeding of membrane and formation of apoptotic bodies [18,19].

In this study, Hoechst 33258 assay was used to monitor changes in the nucleus of HL-60 cells induced after treated with Lp16-PSP. Hoechst 33258 is a DNA-specific fluorochrome which upon excitation with UV emits a blue fluorescence. As shown in Figure. 1C, the nuclei of untreated HL-60 cells are round with homogenous blue fluorescence. Whereas, Lp16-PSP exposed cells showed shrinkage, nuclear chromatin condensation, and apoptotic body formation. The oligonucleosomal fragmentation of chromosomal DNA is another biochemical feature of apoptosis [20-22] that was studied by using DNA fragmentation assay after Lp16-PSP treatment of HL-60 cells for 48 h. DNA was extracted from HL-60 cells and studied using agarose gel electrophoresis. The electrophoretogram given is showing the fragmentation of DNA, while no significant "DNA ladder-like" pattern was found in the control group (Fig. 1D). Moreover, a concentration-dependent increase in DNA cleavage was also observed for the Lp16-PSP treated samples.

Another hallmark of apoptosis is the externalization of phosphatidylserine on the cell membrane prior to the loss of cell membrane integrity [19,23], which can be monitored by annexin V/propidium iodide (AV/PI) staining [24,25]. HL-60 cells after treatment with various concentrations (0 µg/ml, 50 µg/ml, 100 µg/ml and 150 µg/ml) of Lp16-PSP for 48 h were analyzed for the induction of apoptosis by using annexin V/propidium iodide (AV/PI) staining. After 48 h treatment, the percentage of apoptotic cells (including early and late apoptotic cells) increased with the concentration of Lp16-PSP from 3.51 % to 45.61 % (Fig. 1E). Statistical analysis showed that the percentage of cells in late apoptosis stage was significantly higher than the control group upon Lp16-PSP (100 µg/ml and 150 µg/ml) treatment for 48 h. These results suggested that the HL-60 cell death by Lp16-PSP is through the induction of apoptosis.

Figure 1



(a) (b)

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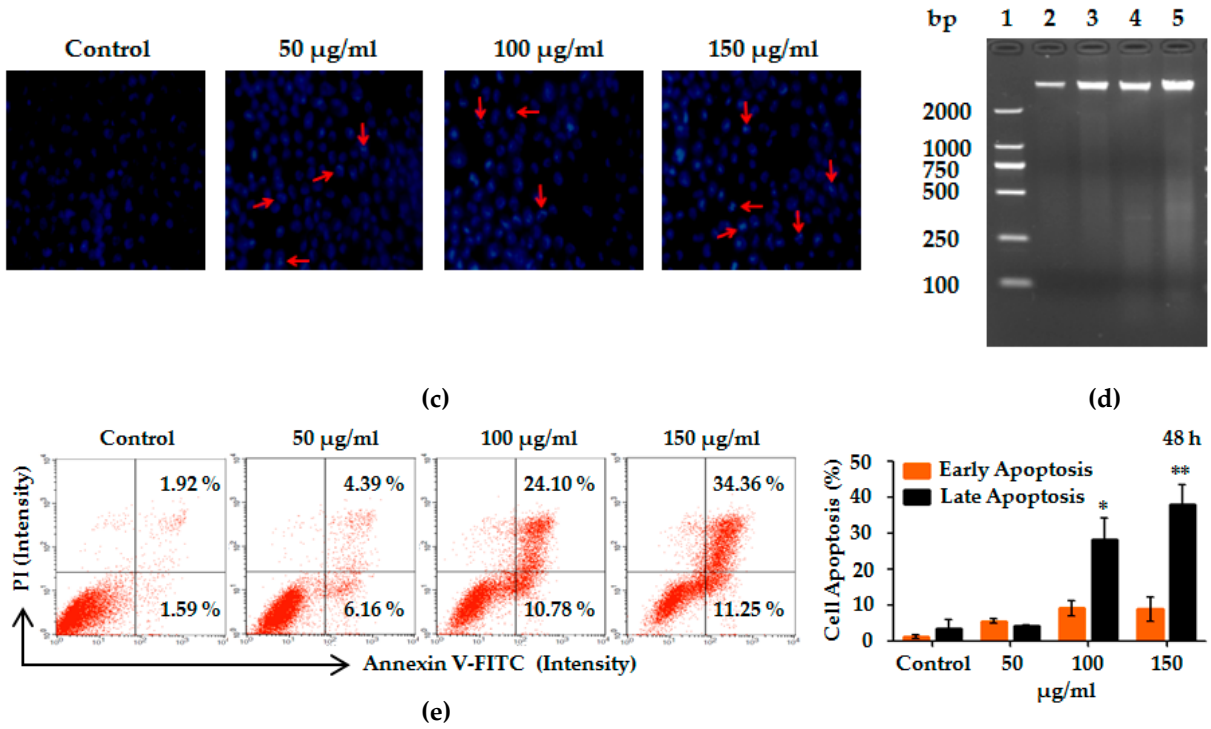

Figure 1: Lp16-PSP-induced apoptosis in acute promyeloid leukemia (HL-60 cells). (a) Phase contrast image of HL-60 cells, untreated and treated samples after 48 h of Lp16-PSP treatment. Red arrowheads are indicating the cells smaller in size and also showing cellular bleeding. **(b)** Effect of different concentration of Lp16-PSP (0 µg/ml, 12.5 µg/ml, 25 µg/ml and 50 µg/ml) on colony formation (anchorage-independent growth) of HL-60 cells was evaluated and a number of colonies were counted as described in Materials and methods. The data reported here is the mean ± SD of two separate experiments, *p<0.05, **p<0.01. **(c)** Fluorescent images of Hoechst 33258 stained HL-60 cells for nuclear morphological changes after 48 h of Lp16-PSP treatment with indicated concentrations. **(d)** DNA fragmentation of HL-60 cells treated with 0 µg/ml, 50 µg/ml, 100 µg/ml and 150 µg/ml of Lp16-PSP for 48 h. Lane 1: DNA marker DL2000, Lane 2: 0 µg/ml, Lane 3: 50 µg/ml, Lane 4: 100 µg/ml, and Lane 5: 150 µg/ml Lp16-PSP exposed group. **(e)** Apoptosis was analyzed by Annexin-V/PI staining after 48 h treatment of HL-60 cells with indicated concentrations of Lp16-PSP. Left, results as histogram from one representative experiment treated with 0 µg/ml, 50 µg/ml, 100 µg/ml and 150 µg/ml of Lp16-PSP after 48 h treatment. The lower left (Annexin V-/PI-) and lower right (Annexin V+/PI-) quadrants are showing the viable cell population and the cells at the early apoptosis respectively, and the upper-right quadrant (Annexin V+/PI+) is showing the cell population at the late apoptosis. Right, a number of cells in early and late apoptotic phase were calculated. The data are expressed as mean ± SD (*p<0.05, **p<0.01).

2.3 Involvement of extrinsic and intrinsic pathways in the Lp16-PSP induced apoptosis: Apoptosis occurs through two main pathways: the Fas death receptor-triggered extrinsic pathway [26] and the mitochondrial-mediated or intrinsic pathway [27]. The initiator caspases i.e. caspase-8 and -9, upon activation, causes the activation of caspase-3, -6, and -7 which results in the cleavage of the cytoskeleton and nuclear protein, ultimately leading to apoptosis [28]. Bcl-2 family of proteins that also play a central role in intrinsic apoptosis pathway by binding with Bax and preventing the mitochondrial pore formation and the release of cytochrome c [29]. On the other hand, pro-apoptotic Bax expression causes the induction of apoptosis [30]. So, in order to characterize the Lp16-PSP induced apoptosis, we evaluated various

apoptosis-related genes such as *Bax*, *Bcl-2*, *Caspase-3*, *Caspase-8*, *Caspase-9*, and *FasL* after treatment with Lp16-PSP by using qRT-PCR. Treatment with Lp16-PSP (IC_{50} concentration) resulted in the significant up- and down-regulation of *FasL* and *Bcl-2* transcripts respectively, together with increased expression of *Bax*, *Caspase-3*, *-8* and *-9* (Fig. 2A, and 3B). Furthermore, the activation of caspase-8 and -9 (initiator caspases) and caspase-3 (effector caspase) was confirmed by the colorimetric assay performed after 48 h of treatment with different concentrations of Lp16-PSP which showed the activation of caspase-8, -9 and -3 in a dose-dependent fashion (Fig. 2B). In addition, western blot analysis also revealed the cleavage of caspase-3 and the dose-dependent increase and decrease in the expression of Bax and Bcl-2 proteins respectively (Fig. 2C). Increased expression of Bax results in the increased cytochrome c release, that is associated with the mitochondrial damage and intrinsic pathway [31]. In our study, Lp16-PSP has resulted in the significant mitochondrial membrane potential loss which ultimately resulted in the release of cytochrome c from mitochondria into the cytosol (Fig. 2D, 2E). All these findings suggest that Lp16-PSP have triggered both extrinsic and intrinsic apoptosis pathway, however, a detailed investigation is required to further characterize the Lp16-PSP induced apoptosis in terms of signal transduction pathway (s) responsible for these outcomes.

Figure 2

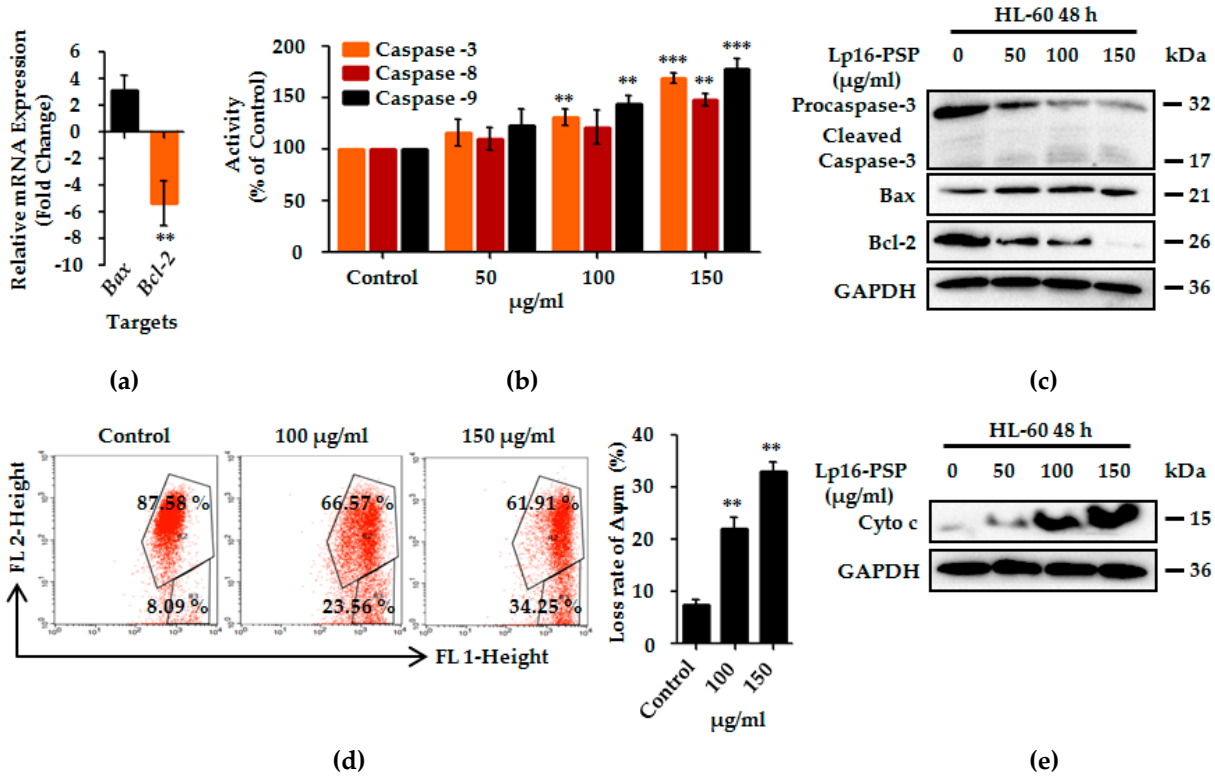

Figure 2: Involvement of Extrinsic and Intrinsic Pathway in Lp16-PSP-induced Apoptosis. (a) Effect of Lp16-PSP on the expression of *Bax* a pro-apoptotic and *Bcl-2* an anti-apoptotic genes, after 48 h of treatment, as evaluated by qRT-PCR. The mRNAs under investigation from the test was normalized to GAPDH and plotted as fold change to the mRNA of control untreated cells, defined as 1. The data expressed here are mean ± SD of the three individual

experiments (**p<0.01). **(b)** The colorimetric analysis of caspase-3, -8, and -9 after treatment with an indicated concentration of Lp16-PSP for 48 h. The data reported here are the mean ± SD of three independent experiments each performed in triplicate (**p<0.01, ***p<0.001). **(c)** Western blot analysis of the cleavage of caspase-3, Bax, and Bcl-2 after treatment with different concentrations (0 μg/ml, 50 μg/ml, 100 μg/ml and 150 μg/ml) of Lp16-PSP for 48 h, using GAPDH as an internal control. **(d)** The loss of mitochondrial membrane potential in HL-60 cells after treatment with indicated concentrations of Lp16-PSP for 48 h. Left, results from one representative experiment of HL-60 cells treated with indicated concentrations of Lp16-PSP. Right, the loss rate of mitochondrial membrane potential as compared with the control. The data reported here are mean ± SD (**p<0.01). **(e)** The release of cytochrome c detected by western blotting after treatment with indicated concentrations of Lp16-PSP for 48 h, using GAPDH as an internal control.

2.4 Lp16-PSP Induces G_1 Phase Cell Cycle Arrest in HL-60 cells: Lp16-PSP resulted in the suppression of HL-60 cell growth in a dose- and time-dependent fashion [15]. In order to verify that the suppression of growth is due to the disruption of the cell cycle, flow cytometry was done for the analysis of cell cycle distribution after Lp16-PSP treatment at different concentrations (0 μg/ml, 25 μg/ml, and 50 μg/ml). As shown in Figure. 3A, Lp16-PSP treatment at low doses i.e. 25 μg/ml or 50 μg/ml resulted in an increased cell population in G_1 phase, with approximately 49 % and 60 % cells in G_1 phase respectively, in comparison to approximately 32 % in control after 48 h treatment. This increase G_1 phase cell population was observed to be related to the decrease in the S phase population, however upon treatment G_2/M phase remained unchanged. These findings suggest that Lp16-PSP at low doses caused HL-60 growth suppression by modulating the progression of cell cycle without the induction of apoptosis.

2.5 Lp16-PSP induced $p21^{WAF1/CIP1}$ mediated G_1 cell cycle arrest in HL-60: Furthermore, we investigated the effect of Lp16-PSP on different cell cycle regulatory gene at mRNA and protein levels after 48 h of treatment. As p21 is well-recognized as the universal inhibitor of cyclin-cdk complexes [32-34] we assessed the expression of p21, and p27, cyclins (cyclin D1, cyclin E1) and cdks (cdk2, cdk4, cdk6) that are operative in the G_1 phase of the cell cycle by qRT-PCR. Treatment of HL-60 cells with Lp16-PSP (IC_{50} concentration) resulted in the down-regulation of *cdk2*, *cdk4*, *cdk6*, *cyclin D1* and *cyclin E1*, with the significant up-regulation of *CDK inhibitory genes* (*p21*), however, increased expression of *p27* was also observed (Fig. 3B). Moreover, Western blotting revealed the dose-dependent decrease and increase in the expression of cdk6, cyclin D1, cyclin E1 and p21 respectively (Fig. 3C). Therefore, these results suggested that proliferation inhibition and G_1 arrest in HL-60 is mediated by the upregulation of p21 that is involved in the progression of the cell cycle from the G_1-S phase.

It has been uncovered by the molecular analysis of human tumors that cell cycle controllers are often mutated in the majority of the malignancies thus the control of cell cycle progression in cancer is thought to be one of the compelling strategies to battle cancer [35,36]. Our data suggested the potential application of Lp16-PSP in combating cancers with deregulated cell cycle components.

Figure 3

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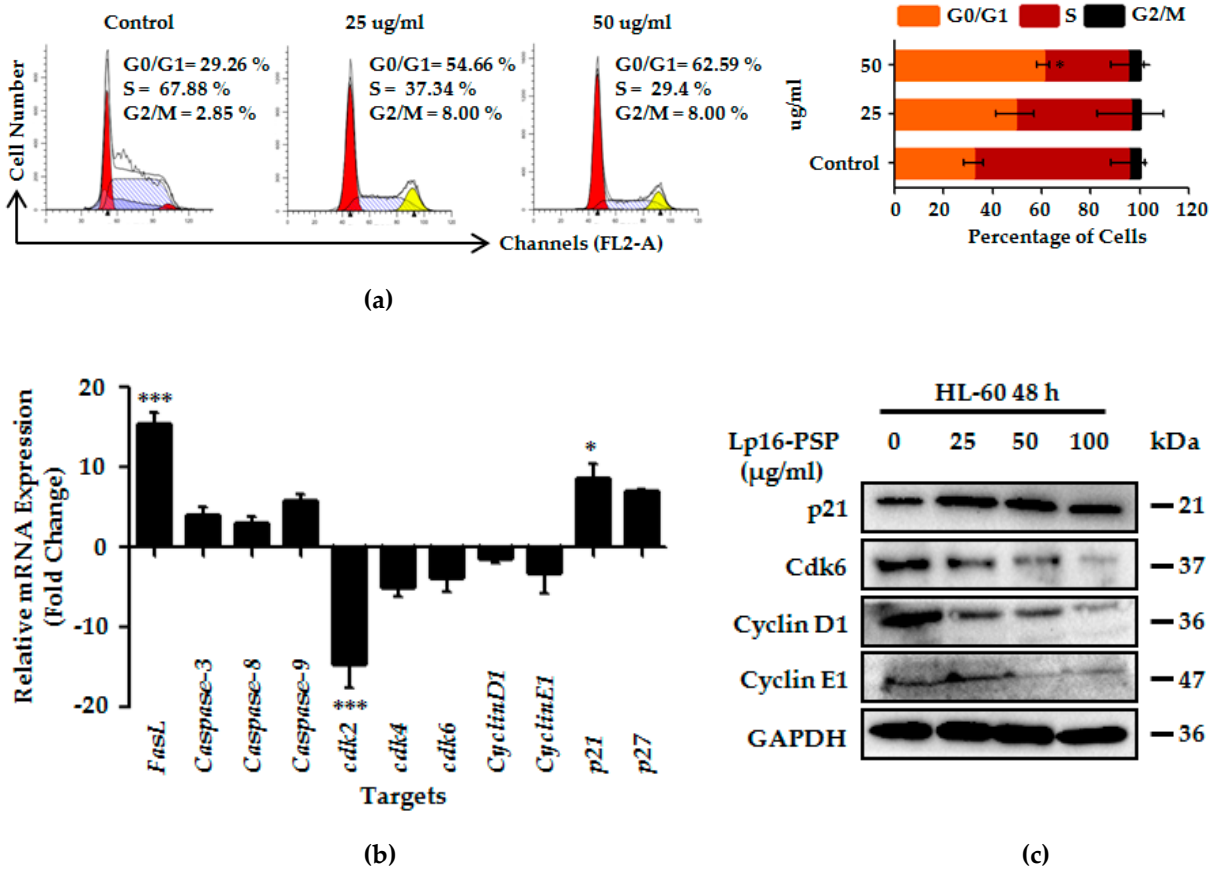

Figure 3: Lp16-PSP-Induced p21$^{WAF1/CIP1}$ mediated G_1 Cell Cycle Arrest in HL-60 Cells. (a) HL-60 cells untreated and treated with various concentrations of Lp16-PSP for 48h were analyzed for DNA content using flow cytometry. Left, results of the one representative experiment indicating the distribution and percentages of cells in G0/G_1, S and G2/M phase. Right, is the graphical presentation of the distribution and percentages of cells in different phases of cell cycle. The data presented here is mean ± SD where *p<0.05. **(b)** qRT-PCR analysis of apoptosis- and cell cycle-related genes. The data presented here is the mean ± SD of two independent experiments each run in triplicate. Where *p<0.05, ***p<0.001. **(c)** Western Blot of cell cycle-related proteins after Lp16-PSP treatment with indicated concentrations for 48 h. GAPDH was used as internal control.

3. Discussion: We have reported the cloning, expression and selective *in vitro* anticancer activity of Lp16-PSP from *L edodes* strain C_{91-3} against a panel of human cancer and normal cell lines and HL-60 cell line was identified as one of the most sensitive cell lines used [15]. So, for further investigations, we have used human acute promyeloid leukemia (HL-60 cells) as model cancer. In this study, our findings demonstrated that high doses of Lp16-PSP resulted in the induction of morphological changes (Fig. 1A), nuclear chromatin condensation (Fig. 1C), cleavage of chromosomal DNA in a DNA ladder-like pattern (Fig. 1D), accumulation of significant percentage of apoptotic cells in the lower right (Annexin V+/PI-) and upper right (Annexin V+/PI+) quadrants (Fig. 1E) and the loss of mitochondrial membrane potential (Fig. 2D) in HL-60 cells.

Initially, the expression of apoptosis- and cell cycle-related genes was evaluated by qRT-PCR. The results showed that Lp16-PSP treatment (IC_{50} concentration) for 48 h resulted in the upregulation of *Bax*, *caspase-3*, *caspase-8*, *caspase-9*, *FasL*, *p21*, and *p27* with the downregulation of *Bcl-2*, *cdk2*, *cdk4*, *cdk6*, *cyclin D1*, and *cyclin E1* transcripts in HL-60 cells (Fig. 2A, 3B). Activation of the initiator (caspase-8, caspase-9) and the effector caspases (caspase-3) was confirmed by colorimetric analysis, where Lp16-PSP resulted in the dose-dependent increase in the activity of caspase-3, -8 and -9 (Fig. 2B). Increased expression of FasL and activation of caspase-8 clearly demonstrated that extrinsic pathway might be involved in Lp16-PSP induced apoptosis. Furthermore, western blot analysis revealed the cleavage of caspase-3, increased expression of Bax, Bcl-2, and release of cytochrome c after treatment with Lp16-PSP in a dose-dependent fashion (Fig. 2C and 2E). These findings suggested the involvement of intrinsic pathway in Lp16-PSP induced apoptosis in HL-60 cells.

Low doses of Lp16-PSP resulted in the anchorage-independent growth inhibition (Fig. 1B) and the induction of G_1 cell cycle arrest as demonstrated by the results of flow cytometry (Fig. 3A). In addition, increased expression of the universal inhibitor of cyclin-cdk complexes ($p21^{WAF1/CIP1}$) together with the decreased expression of cyclin D, E, and cdk6 was also confirmed by western blot analysis (Fig. 3C). These findings suggest that Lp16-PSP resulted in the induction of $p21^{WAF1/CIP1}$ mediated G_1 cell cycle arrest in HL-60 cells. Several studies have shown that induction of apoptosis and cell cycle arrest are the valuable strategies for cancer drug discovery [37-39]. However, at this stage, it's difficult to conclude the possible molecular mechanism of the action of Lp16-PSP and further support our experimental findings on the basis of previously reported information associated with the other members of YjgF/YER057c/UK114 family. Although antineoplastic, ribonuclease, inhibition of protein synthesis and antiviral activities of the other members of YjgF/YER057c/UK114 protein family has been reported [40-45], and afterward it was proven that translation inhibition was driven by endoribonucleolytic activity. In addition, various ribonucleases have also been reported to have selective anticancer properties [46-50]. Based on previously available information related to YjgF/YER057c/UK114 family members and our findings in this study, we believe that Lp16-PSP might have exerted it's *in vitro* anticancer activity through RNA (s) degradation and/or through the inhibition of protein synthesis. However, this study gives a very preliminary indication of the potential application of Lp16-PSP in combating cancer. Thus, further investigations are needed to overcome the shortcomings of this study including the *in vitro* enzymatic (endoribonuclease) activity of Lp16-PSP, substrate/target identification (tRNA, rRNA, mRNA, microRNA or lncRNA), mode of entry into the cell (specific receptors mediated endocytosis), resistance to ribonuclease inhibitors, site of action (nucleus/cytosol), interaction with intracellular molecules, so that the detailed molecular mechanism in both *in vitro* and *in vivo* models can be explored, activity of Lp16-PSP can be compared with the already known antitumor ribonucleases and most important therapeutic implication of Lp16-PSP can be made possible in near future after concrete preclinical and clinical trials.

4. Material and Methods:

Expression of the Recombinant Protein Lp16-PSP: The Latcripin-16 (designated as Lp16-PSP) is one of the registered proteins of *Lentinula edodes* C_{91-3} from our laboratory, with the accession # AHB81541. The expression and recovery of the bioactive form of 32 kDa Lp16-PSP protein were accompanied as described

previously [15]. Briefly, for routine experimentation, Lp16-PSP was expressed at 37 °C after induction with 0.5 mM IPTG for 4 h, in Rosetta gami (DE3), using pET32a (+) as the expression vector. Solubilization of the protein was achieved by mild solubilization buffer containing 2 M urea by the freeze-thaw method. Purification and refolding were done under optimized conditions. The finalized protein thus obtained after extensive dialysis was concentrated by using PEG 20,000. At each step, Lp16-PSP was qualitatively and quantitatively analyzed by SDS-PAGE and BCA respectively and then used subsequently for biological assays.

Human Leukemia HL-60 Cells and Culture Conditions: Human acute promyeloid leukemia cell line (HL-60) was obtained from Shanghai cell bank, Chinese Academy of Sciences (Shanghai, China) and was growth in RPMI medium containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37 °C in a humidified atmosphere containing 5 % CO_2. HL-60 cells were maintained in exponential growth, and they were passaged when cell confluency reached ~ 80 %.

Antibodies, Kits, and Reagents: Bax, Bcl-2, caspase-3, cdk6, cytochrome c, cyclin D1, cyclin E1, GAPDH, p21, secondary antibodies and RIPA buffer were from Proteintech (China). The Annexin-V/PI Kit, Mitochondrial membrane potential kit, Hoechst 33258 Assay Kit and DNA fragmentation kit, Caspase-3, -8 and -9 kits were purchased from keyGEN BioTECH (Nanjing, China). The qRT-PCR kit was purchased from Transgene (China). All other reagents and chemicals were purchased from standard commercial sources.

Phase Contrast Imaging: The HL-60 cells (2 x 10^5 cells/well) after overnight incubation at 37 °C in 12 well plate was washed with PBS once and grown for 48 h in culture media with previously established doses of Lp16-PSP (0 µg/ml, 50 µg/ml, 100 µg/ml and 150 µg/ml). After treatment cell morphology was examined and photographed by using phase contrast microscope [51].

Soft-agar Colony Formation Assay: The evaluation of anchorage-independent growth was done by clonogenicity of cells on soft-agar. Lp16-PSP treated (0 µg/ml, 12.5 µg/ml, 25 µg/ml and 50 µg/ml) HL-60 were mixed with 1.2 % agar in growth medium, and plated on top of a solidified layer of 0.3% agar in growth medium, in 6 well plates. Cells were fed every 3 days with growth medium, and colony formation was observed daily under a phase-contrast microscope. A number of colonies were counted in five fields under a microscope at 40 x magnification [52].

Hoechst 33258 Staining: HL-60 cells (2 x 10^5 cells/well) in 12 well plate after treatment with indicated concentrations of Lp16-PSP for 48 h were washed twice with cold buffer A provided with the kit (Hoechst 33258 Detection Kit, Keygen, China) and fixed by using 4 % formaldehyde solution at 4 °C for 10 min. Cells were washed again with buffer A and stained with 100 µl of Hoechst 33258 working solution for 10 min at room temperature. After washing with water and air dry, cells were observed with a fluorescence microscope.

DNA Fragmentation Assay: HL-60 cells (5 x 10^6 cells) were treated with different concentration of Lp16-PSP (0 µg/ml, 50 µg/ml, 100 µg/ml, and 150 µg/ml), and after 48 h of treatment DNA fragmentation assay was done following the manufacturer's instructions (DNA Fragmentation Assay Kit KeyGen, China). Briefly, cells after treatment were collected in 1.5 E.P tubes and washed with PBS. Cells were lysed with

the lysis buffer and enzymes provided with the kit, DNA was then precipitated and washed with 70 % ethanol. 10 µL of DNA samples were mixed with the loading buffer, run on 1.5 % agarose gel and image was captured by a ChemiDco™ XRS + Imager-Bio-Rad.

Colorimetric Analysis of Caspase-3, -8 and -9: Caspase activities in HL-60 cells after treatment with indicated concentrations of Lp16-PSP for 48 h, were measured by using the commercially available kits (Caspase-3, -8 and -9 Kit KeyGen, China). Briefly, cells after treatment with several concentrations of Lp16-PSP for 48 h, were washed twice with PBS and subjected to caspase assay as per manufacturer's instructions. The activity of the caspase-3, -8 and -9 was normalized and expressed as $O.D_{Test}$ /$O.D_{Control}$ x 100.

Apoptosis Analysis using Annexin-V-FITC/PI Staining: HL-60 cells were treated with different concentrations of Lp16-PSP for 48 h. Thereafter, cells were collected, washed and stained as per manufacturer's instructions (Apoptosis Detection Kit Keygen, China). The rate of apoptosis was measured by flow cytometry (FACS-Calibur Cytometer (BD Biosciences, Heidelberg, Germany)) within 1 h.

Mitochondrial Membrane Potential ($\Delta\psi m$) Measurement using JC-1 staining by Flow Cytometry: Mitochondrial membrane potential assay was performed as per manufacturer's instructions. Briefly, HL-60 cells after treatment with Lp16-PSP (0 µg/ml, 100 µg/ml, and 150 µg/ml) for 48 h were collected after centrifugation and washed twice with PBS. Cells were then incubated with the working solution of JC-1 stain at 37 °C for 30 min. Cells were then collected and resuspended in incubation buffer provided with the kit, and loss of mitochondrial membrane potential was analyzed by using a FACS-Calibur Cytometer (BD Biosciences, Heidelberg, Germany).

Cell-Cycle Analysis by Flow Cytometry: HL-60 cells were treated with indicated concentrations of Lp16-PSP for 48 h. After treatment cells were collected, washed with PBS and fixed overnight with 70 % ethanol at 4 °C. After fixing, cells were collected by slow centrifugation, washed with ice-cold PBS and resuspended at a concentration of 1 x 10^6 cells/ml in 5 µg/ml RNase and 50 µg/ml propidium iodide. The cells were then incubated for 30 min at 37 °C and analyzed by using a FACS-Calibur Cytometer (BD Biosciences, Heidelberg, Germany).

Isolation of RNA and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR): Expression of the apoptosis- and cell cycle-related genes was determined by quantitative real-time PCR. After treatment of the HL-60 cells with IC_{50} concentration for 48 h, total RNA was extracted from Lp16-PSP treated and control cells with TRIzol reagent (Life Technology), according to the manufacturers' instructions. One microgram of RNA was used to generate cDNA by using Transgene RT reagent Kit with gDNA remover. To quantify a number of transcripts, SYBER Green based qPCR was performed with RT master mix (Transgene) using Real-Time PCR System (StepOne™). The thermal profile used was as follows: For Reverse transcription 42 °C - 15 min, 85 °C - 5 s, for quantitative PCR 94 °C - 30 s, 40 x [94 °C - 5 s, 60 °C - 15 s, 72 °C - 10 s]. The primer sequences for apoptosis- and cell cycle-related genes are listed in (Table.1). GAPDH was used as internal control and all the reactions were performed in triplicate. The relative gene expression was calculated by using the $2^{-\Delta\Delta C_T}$ method as described previously [53].

Table 1: qRT-PCR Primer Sequences

Gene (s)	Forward 5′ → 3′	Reverse 5′ → 3′
Bax	CCCGAGAGGTCTTTTTCCGAG	CCAGCCCATGATGGTTCTGAT
Bcl-2	GGTGGGGTCATGTGTGTGG	CGGTTCAGGTACTCAGTCATCC
Cas3	AGAGGGGATCGTTGTAGAAGTC	ACAGTCCAGTTCTGTACCACG
Cas8	TTTCTGCCTACAGGGTCATGC	TGTCCAACTTTCCTTCTCCCA
Cas9	CTCAGACCAGAGATTCGCAAAC	GCATTTCCCCTCAAACTCTCAA
Cdk2	GCCATTCTCATCGGGTCCTC	ATTTGCAGCCCAGGAGGATT
Cdk4	ATGGCTACCTCTCGATATGAGC	CATTGGGGACTCTCACACTCT
Cdk6	CTGCAGGGAAAGAAAAGTGC	CTCCTCGAAGCGAAGTCCTC
Cyclin D1	GCTGCGAAGTGGAAACCAT	CCTCCTTCTGCACACATTTGAA
Cyclin E1	AAGGAGCGGGACACCATGA	ACGGTCACGTTTGCCTTCC
FasL	TGCCTTGGTAGGATTGGGC	GCTGGTAGACTCTCGGAGTTC
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA
p21	TGTCCGTCAGAACCCATGC	AAAGTCGAAGTTCCATCGCTC
p27	TAATTGGGGCTCCGGCTAACT	TGCAGGTCGCTTCCTTATTCC

Western Blotting: Lp16-PSP treated and control cells were lysed in RIPA buffer supplemented with protease inhibitors. Cell lysates were cleared by centrifugation at 14,000 rpm, for 20 min at 4 °C and proteins (20 – 40 μg) were resolved by electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. In order to prevent the non-specific antibody binding, membranes were blocked with blocking buffer (5 % skimmed milk in TBS-T (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 % Tween 20)) at room temperature for 1 h. Blots were incubated at 4 °C overnight with the following antibodies diluted with blocking buffer: Bax (Cat # 23931-1-AP, 1:500), Bcl-2 (Cat # 12789-1-AP, 1:1000), caspase-3 (Cat # 19677-1-AP, 1:200), cyclin D1 (Cat # 60186-1-AP, 1:2000), cyclin E1 (Cat # 11554-1-AP, 1:500), cdk6 (Cat # 14052-1-AP, 1:200), cytochrome c (Cat # 10993-1-AP, 1:200), GAPDH (Cat # 23931-1-AP, 1:500), p21 (Cat # 10355-1-AP, 1:500). Next membranes were incubated with the Second antibodies: Goat anti-rabbit IgG, Goat anti-mouse IgG (HRP-conjugated, Proteintech) (Cat # 23931-1-AP, SA00001-1, 1:500) at room temperature for 1 h. Blots were developed with ECL chemiluminescence detection kit and images were captured by a ChemiDco™ XRS + Imager-Bio-Rad.

Statistical Evaluation: Statistical analysis was done by using GraphPad Prism 5.0 software (La Jolla, CA, USA). All the experiments were done in triplicate unless otherwise stated. Data were evaluated for significance by using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test.

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