Alternative Therapeutic Approach to Urothelial Cell Carcinoma with Medicinal Mushroom Extracts

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Abstract   Severe side effects from chemotherapy or immunotherapy often limit their clinical utility in urothelial cell carcinoma of the bladder. To explore alternative therapies, we were particularly interested in one of medicinal mushrooms called *Phellinus linteus* (PL) with antitumor/anticancer activity. We investigated possible anticancer effects of three distinct extracts or fractions of PL using an urothelial cell carcinoma (UCC) model *in vitro*. Those fractions included PL-I (crude extract), PL-II (water-extracted), and PL-III (ethanol-extracted), which were tested for their antiproliferative effects on UCC cells. To explore the anticancer mechanism(s) of these fractions, cell cycle analysis and analyses of epigenetic parameters, caspases-3 and -9, and apoptotic regulators, were also performed. Both PL-I and -II induced a maximum growth reduction of ~60% at 700 µg/ml while PL-III led to a ~90% growth reduction at 150 µg/ml in 72 h. Cell cycle analysis indicated that cells treated with 500 µg/ml (IC_{50}) of PL-I and -II or 60 µg/ml (IC_{50}) of PL-III underwent a G_1 cell cycle arrest, accompanied by the up-regulation of p21, a cell cycle-dependent kinase inhibitor. Additionally, histone deacetylase (HDAC) activity was significantly (>60%) lost, while both histones H3 and H4 were highly acetylated, indicating alterations in the chromatin structure. Moreover, both caspases-3 and -9 in cells treated with each IC_{50} of three PL-fractions were all significantly (p<0.03) activated, implying induction of apoptosis. The present study shows that all three PL-fractions, PL-I, -II, and -III, have anticancer effects on UCC cells, although PL-III appears to be the most potent. Such an anticancer mechanism is attributed to a p21-mediated cell cycle arrest with epigenetic modifications involving HDAC inactivation and hyperacetylation of H3 and H4, ultimately leading to apoptotic cell death. Therefore, PL-fractions may have clinical implications in a safer and improved therapeutic modality for urothelial cell carcinoma.

Keywords: mushrooms, Phellinus linteus, cell cycle, histones, urothelial cell carcinoma

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1. Introduction

Urothelial cell carcinoma (UCC) of the bladder is a growing epidemic and a major global health challenge with an estimated 429,000 new cases and 165,000 deaths annually worldwide [1]. In the United States alone, approximately 75,000 new cases were diagnosed and nearly 16,000 patients died from bladder cancer in 2014 [2]. Over the past 20 years, the 5-year survival rates of UCC have been 33% and 5% for locally advanced and metastatic disease, respectively [2]. This is a serious challenge for urgently establishing the improved treatment modality.

The current therapeutic options for UCC, whether muscle invasive or non-muscle invasive, are limited. For non-muscle invasive bladder cancer the therapies are limited to endoscopic transurethral resection (TUR) with or without intravesical chemotherapy or immunotherapy. However, 60%-70% of patients will recur and approximately 25% will progress to muscle invasive disease within 5 years [3], becoming untreatable and fatal. Although the two major treatment modalities for muscle invasive disease are chemotherapy and surgery, the

prognosis following such treatments shows the 5-year survival rate of only 60%, due to distant recurrence [4]. Thus, it is crucial to prevent multiple recurrences and progression to a more advanced, invasive disease. Nevertheless, it should be also noted that inevitable, palpable side effects from chemotherapy or immunotherapy are another drawback. Over the past several decades, only limited improvement or progress has been made in UCC therapy. There is the urgent need for a more effective and safer treatment modality with few side effects.

Recently, the medicinal aspects of various natural agents and substances have gained more public attention. Those include herbs, mushrooms, flowers, fruits, plant seeds, sea weeds, algae, tea, bark, shark cartilage and so on. We were particularly interested in one well-established medicinal mushroom called *Phellinus linteus* (PL). PL has been used in Asian countries for centuries to prevent/treat ailments such as gastroenteric dysfunction, diarrhea, hemorrhage, rheumatoid arthritis, and cancers [5]. To develop it for anticancer therapeutics, various "PL-extracts or fractions" have been isolated and intensively studied. A number of studies revealed that they had the antitumor, immunomodulatory, anti-angiogenic, and antioxidant properties [6,7,8,9]. In fact, antitumor or anticancer activities

of these extracts have been demonstrated in a variety of cancers and tumors *in vitro* and *in vivo*, including prostate cancer, lung cancer, colon cancer, breast cancer, melanoma, and leukemia [10-16]. It is thus plausible that such antitumor/anticancer effects of PL-fractions are the potential area for developing novel therapies for UCC.

Accordingly, we investigated if three distinct PL-fractions, *PL-I, PL-II,* and *PL-III*, might have potential anticancer effects on human bladder cancer cells *in vitro*. To explore the anticancer mechanism of these fractions, our study focused on the cell cycle regulation, chromatin modifications, and induction of apoptosis. To our knowledge, this is one of the first studies on the potential benefits of PL against UCC. More details are described and the significant findings are also discussed herein.

2. Materials and Methods

2.1. Cell Culture

The three types of human bladder cancer cell lines, 5637, T24 and UM-UC3, were obtained from the American Type Culture Collection (Manassas, VA). They are high-grade bladder cancer cells, with the different molecular profiles, derived from different pathological stages (T2-T4) of muscle-invasive urinary bladder cancers [17]. All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Routinely, culture medium was changed every 3 to 4 days and the passage of cells was performed weekly. The three PL-fractions, PL-I (crude extract), PL-II (water-extracted), and PL-III (ethanol-extracted), were a gift from the manufacturer (Mushroom Wisdom, Inc., East Rutherford, NJ). For experiments, cells were seeded in the 6-well plates or T-75 culture flasks at the initial cell density of 2×10^5 cells/ml and treated with varying concentrations of three PL-fractions. Cell morphology was monitored everyday and cell growth/viability was determined by MTT assay.

2.2. MTT Assay

Cell growth/viability was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay following the vendor's protocol (Sigma-Aldrich, St. Louis, MO). Briefly, at the harvest time, 1 ml of MTT reagent (1 mg/ml) was added to each well in the 6-well plate, followed by 3-h incubation at 37°C. After removing MTT reagent, dimethyl sulfoxide (DMSO) was added to each well and absorbance of formazan solution (purple) was read on a microplate reader. Cell viability was determined and expressed by the percent (%) of viable cells relative to the control reading (100%).

2.3. Cell Cycle Analysis

A BD FACscan flow cytometer (Franklin Lakes, NJ), equipped with a double discrimination module, was employed for cell cycle analysis. Approximately 1×10^6 cells were resuspended in 500 μl of propidium iodide solution and incubated at room temperature for 1 h. Ten thousand nuclei were analyzed for each sample, quantified in cell cycle compartments, and estimated as cell cycle phase fractions.

2.4. Histone Deacetylase (HDAC) Assay

HDAC activity was determined using the Epigenase HDAC Assay Kit following the manufacturer's protocol (Epigentek, Farmingdale, NY). Briefly, 10 μg of nuclear extracts from each sample were added to the coated microplate and incubated for 90 min at 37°C. The plate was incubated with the 1st antibody for 60 min, followed by 30-min incubation with the 2nd antibody. The reaction was initiated by the addition of reaction solution, run for 10 min, and terminated with stop solution. Absorbance of all samples was read on a microplate reader, and HDAC activity was calculated and expressed by the % relative to the control reading (100%).

2.5. Western Blot Analysis

Briefly, an equal amount of cell lysates (10 μg), obtained from control and agent-treated cells, was first subjected to 10% SDS gel electrophoresis and transferred to a nitrocellulose membrane (blot). The blot was incubated for 90 min with the primary antibodies against CDK2, CDK4, p21[WAF1], or p27[Kip1] (Santa Cruz Biotechnology, Santa Cruz, CA), followed by 30-min incubation with the secondary antibody conjugates. The specific immune-reactive protein bands were then detected by chemiluminescence following the manufacturer's protocol (KPL, Gaithersburg, MD).

2.6. Enzymatic Assays for Caspase-3 and -9

Enzymatic activities of caspase-3 (Csp-3) and caspase-9 (Csp-9) were determined by the Csp-3 and Csp-9 Colorimetric Assay Kits (BioVision, Milpitas, CA), respectively. Procedures followed as described in the manufacturer's protocol. After control or treated cells (~2 $\times 10^6$ cells) were lysed in lysis buffer, the supernatant (cell lysate) was collected by centrifugation at 13,000 rpm for 10 min and protein concentration was determined. An equal amount (100 μg) of cell lysates was added to the reaction buffer in the 96-well microplate, containing dithiothreitol and the substrate, *either* DEVD-pNA (for Csp-3) *or* LEHD-pNA (for Csp-9), and incubated at 37°C for 2 h. The plate was read at absorbance at 405 nm on a microplate reader. Fold-increase in Csp-3/9 activities was calculated by comparing the readings of treated samples with that of the respective control reading.

2.7. Statistical Analysis

All data were presented as mean ± SD (standard deviation), and statistical differences between groups were assessed with either the unpaired Student's *t* test or one-way analysis of variance (ANOVA). Values of p<0.05 were considered to indicate statistical significance.

3. Results

3.1. Anticancer Effects of Three PL-Fractions on Bladder Cancer Cells

To assess anticancer effects of PL-I, PL-II or PL-III, three bladder cancer cell lines, 5637, T24, and UM-UC3, were separately treated with these three PL-fractions. Cell viability was then determined in 72 h by MTT assay. Overall, all PL-fractions had the significant anticancer

effects on all three bladder cancer cells (Figure 1A, Figure 1B, Figure 1C). Yet, PL-III appeared to be the most potent fraction because it required the less or lower concentration (≥20 µg/ml) to be effective (Figure 1C). To exhibit the same/similar effectiveness of PL-III, both PL-I and PL-II required the higher concentrations, ≥200 µg/ml and ≥100 µg/ml, respectively (Figure 1A, Figure 1B). Thus, these

results show that all three PL-fractions have significant anticancer activities but particularly PL-III is more potent than the rest of two fractions. In addition, among three cancer cells tested, 5637 cells appeared to be most responsive or susceptible to all three PL-fractions at given concentrations. Hence, 5637 cells were further used as a suitable model for the rest of our study.
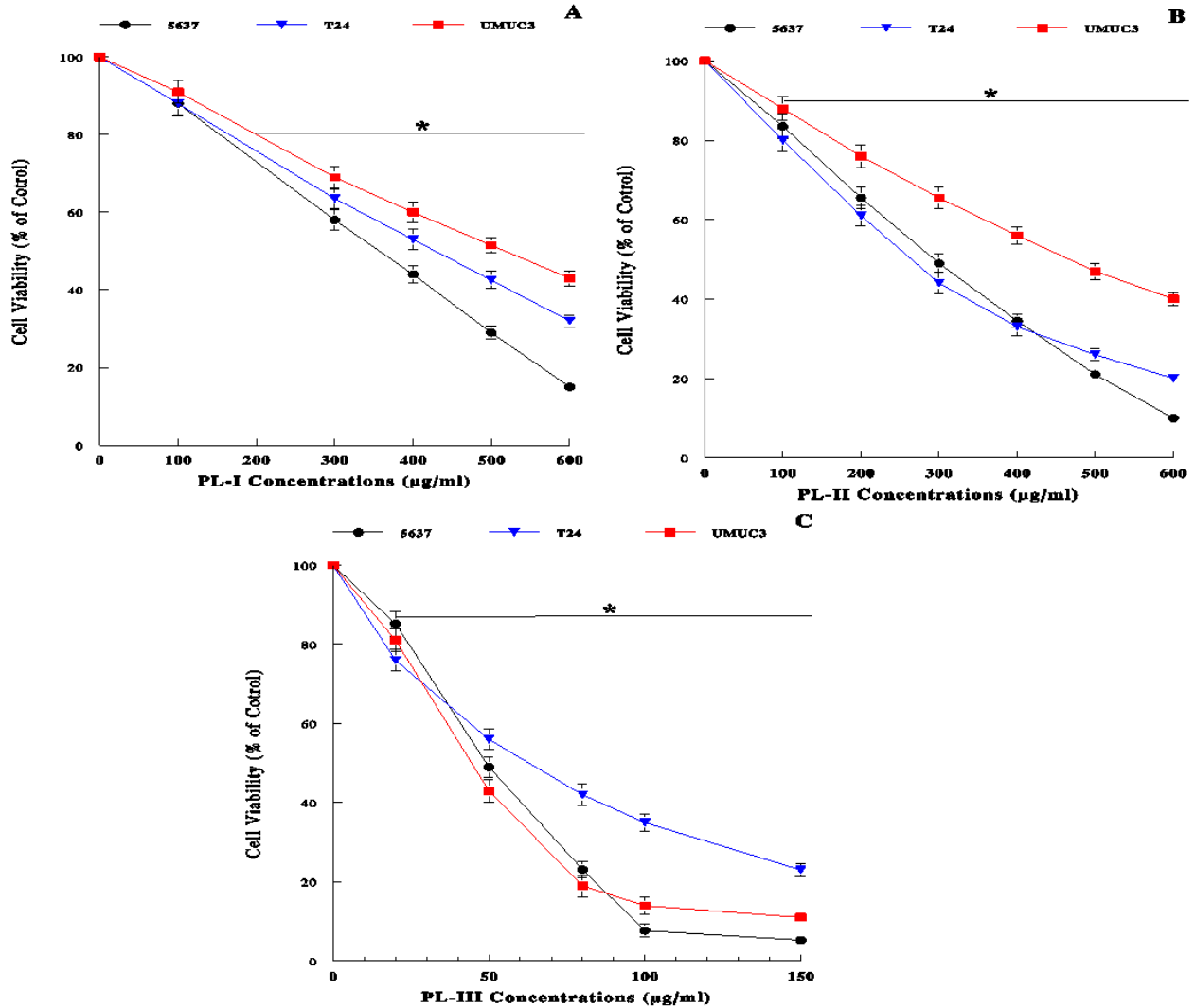

Figure 1. Effects of three PL-fractions on cell viability of bladder cancer cells. Three bladder cancer cells, 5637, T24, and UM-UC3, were treated with indicated concentrations of PL-I (A), PL-II (B), and PL-III (C) for 72 h. Cell viability was determined by MTT assay and expressed by the percent (%) of viable cells relative to controls (100%). All data were calculated as mean ± SD (standard deviation) from three separate experiments. The data points *below* the line with an asterisk in the three panels (A-C) are statistically different (*$p < 0.05$ compared with respective control)
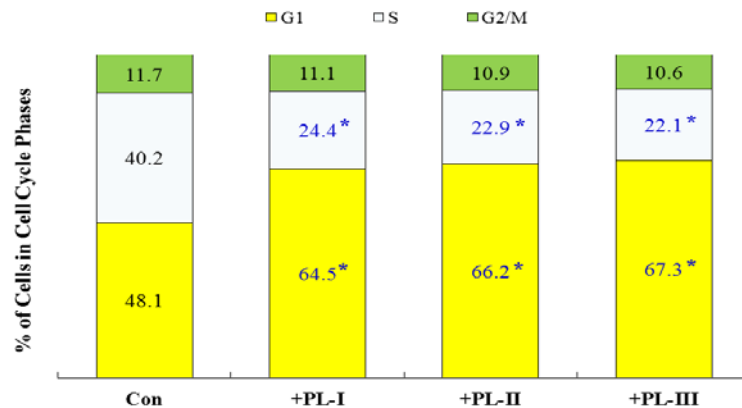

Figure 2. Cell cycle analysis. 5637 cells were treated with PL-I (400 µg/ml), PL-II (300 µg/ml), or PL-III (50 µg/ml) for 72 h and subjected to cell cycle analysis. The % of cells in each cell cycle phase (G_1, S, and G_2/M) under experimental conditions is plotted. All data are mean ± SD from three independent experiments but only the mean values are shown (*$p < 0.05$ compared with respective control)

3.2. Effects of PL-Fractions on Cell Cycle

To explore how these PL-fractions would induce a significant cell viability reduction, 5637 cells were treated with PL-I (400 µg/ml), PL-II (300 µg/ml), or PL-III (50 µg/ml) for 72 h and subjected to cell cycle analysis. These concentrations used were nearly the IC_{50} (50% inhibitory concentration) of each PL-fraction for 5637 cells (Figure 1, Figure 2, Figure 3). The results showed that all three fractions led to a significant (34-40%; p<0.03) *increase* in G_1-phase cell number concomitant with a significant (39-45%; p<0.03) *decrease* in S-phase cell number in 5637 cells (Figure 2). This cell accumulation in the G_1 phase, due to a blockade of the cell cycle transition from the G_1 to the S phase, is known as a G_1 cell cycle arrest [18]. Thus, PL-fractions appear to specifically halt the G_1-S phase progression, subsequently leading to the cell growth/viability reduction.

3.3. Effects of PL-Fractions on G_1 Cell Cycle Regulators

To confirm a G_1 cell cycle arrest induced by PL-fractions, the specific cell cycle regulators for the G_1-S phase transition were also examined. After 5637 cells were treated with PL-fractions (at the concentrations indicated above) for 72 h, four specific regulators, CDK2, CDK4, $p21^{WAF1}$, and $p27^{Kip1}$, were analyzed using Western blots. Such analysis revealed that compared to controls, the expressions of CDK2 and CDK4 were significantly *reduced* or *down-regulated* while those of $p21^{WAF1}$ and $p27^{Kip1}$, the G_1-specific CDK inhibitors, were significantly *enhanced* or *up-regulated* with *all* three PL-fractions. However, *only* the results of PL-III are shown here as a representative (Figure 3). The down-regulation of CDK2 and CDK4, concomitant with the up-regulation of $p21^{WAF1}$ and $p27^{Kip1}$, is clearly indicative of a G_1 cell cycle arrest [19]. Thus, these results further support the notion that PL-fractions may induce a G_1 cell cycle arrest, leading to a growth cessation and consequently the cell viability reduction.
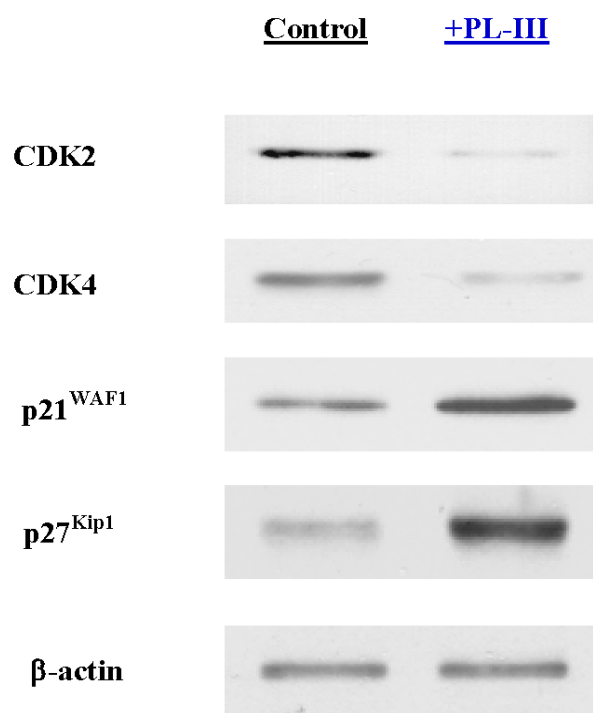

Figure 3. Effects of PL-fractions on G_1 cell cycle regulators. 5637 cells were treated with PL-I (400 µg/ml), PL-II (300 µg/ml), or PL-III (50 µg/ml) for 72 h. The expressions of four G_1 cell cycle regulators, CDK2, CDK4, $p21^{WAF1}$, and $p27^{Kip1}$, were analyzed by Western blots. Although exactly the same expression patterns of these four regulators were observed with treatment of all three fractions, only autoradiographs of four regulators seen with PL-III are shown here. Additionally, β-actin is shown as a protein loading control
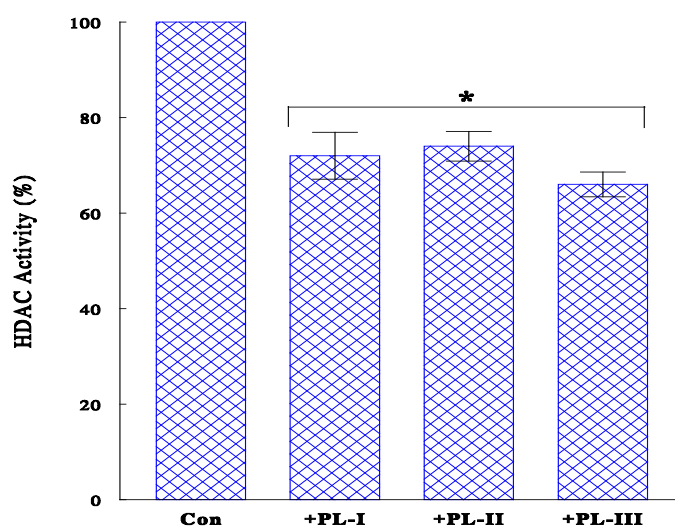

Figure 4. Effects of PL-fractions on HDAC activity. 5637 cells were treated with PL-I (400 µg/ml), PL-II (300 µg/ml), or PL-III (50 µg/ml) for 24 h and subjected to HDAC assay. HDAC activity in cells treated with each fraction is expressed by the % relative to controls (100%). All data were calculated as mean ± SD from three separate experiments (*p<0.05 compared with control)

3.4. Effects of PL-Fractions on Histone Deacetylase (HDAC) Activity

The finding of the up-regulation of $p21^{WAF1}$ with the cell viability reduction (by PL-fractions) was rather interesting because $p21^{WAF1}$ is also known to be regulated by chromatin modifications due to alterations in the acetylation state of histones [20,21]. This possibility was then tested on histone deacetylase (HDAC), one of the primary regulators of histone acetylation [22]. Cells were

first treated with three PL-fractions at the above indicated concentrations for 24 h and subjected to HDAC assay. Such assays revealed that HDAC activity was reduced or diminished by ~28%, ~26%, and ~34% with PL-I, PL-II, and PL-III, respectively (Figure 4). Therefore, these results suggest that PL-fractions may significantly (p<0.05) inactivate HDAC, presumably inducing hyperacetylation of certain histones [20] that could then up-regulate p21^{WAF1} as seen earlier (Figure 3).

3.5. Possible Induction of Apoptosis by PL-Fractions

As it is critical to address if the resulting cell viability reduction (by PL-fractions) would be attributed to apoptosis, this possibility was further explored. Cells treated with three PL-fractions at the indicated concentrations were subjected to enzymatic assays for Csp-3 and Csp-9, the specific apoptotic parameters [23]. Such results showed that *all* three fractions were capable of significantly (p<0.03) activating both Csp-3 and Csp-9 by 2.4-3.0 and 3.0-3.7 folds, respectively, compared to those in control cells (Figure 5). As Csp-3/9 play a central role in apoptosis and act as the positive or pro-apoptotic regulators, their greater activities would rather induce or promote apoptosis [23,24]. Thus, these findings suggest that the cell viability reduction induced by three PL-fractions could be at least in part attributed to apoptosis.
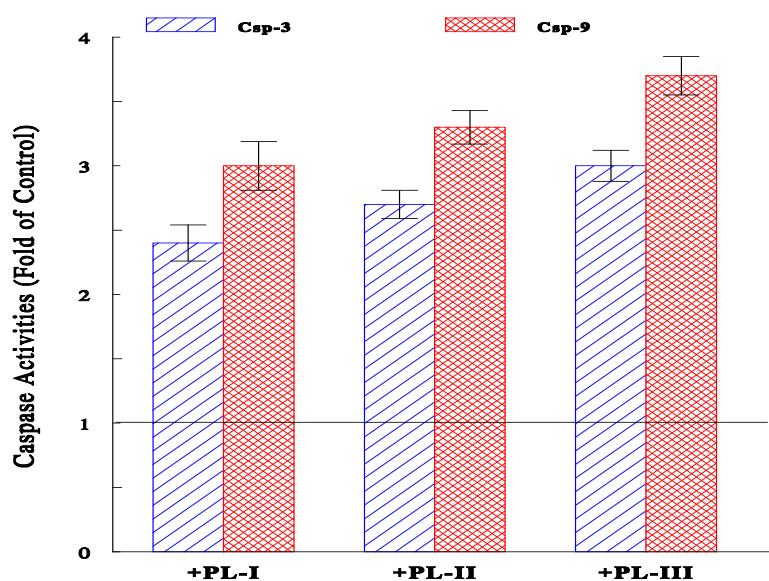

Figure 5. Induction of apoptosis by PL-fractions. 5637 cells were treated with PL-I (400 µg/ml), PL-II (300 µg/ml), or PL-III (50 µg/ml) for 72 h and subjected to enzymatic assays for Csp-3 and Csp-9. Activities of Csp-3 and Csp-9 in cells treated with each fraction were separately determined and expressed by a fold-increase relative to the controls (1.0) as indicated by a line. The data were calculated as mean ± SD from three independent experiments and all three fractions significantly activated both Csp-3 and Csp-9 (p<0.03 compared with respective control)

4. Discussion

Due to the ineffective therapeutic options and unsatisfactory outcomes, patients with UCC and their families are desperately seeking for a more effective therapeutic modality. We have been exploring an alternative way to improve the therapeutic efficacy using natural substances or products. Among them, the three bioactive mushroom fractions isolated from *Phellinus linteus* (PL), PL-I, PL-II, and PL-III, were studied here to find if they would have anticancer effects on human bladder cancer cells *in vitro*.

All three fractions had the significant anticancer effects on the three types of bladder cancer cells, 5637, T24, and UM-UC3 (Figure 1A, Figure 1B, Figure 1C). However, PL-III appears to be the most potent fraction (≥20 µg/ml) to be effective, compared to PL-I (≥200 µg/ml) and PL-II (≥100 µg/ml). As far as a susceptibility of cells to these fractions is concerned, 5637 cells are relatively more susceptible than T24 or UM-UC3 cells and suitable to be used in the rest of our study.

To have an insight into how these fractions would induce a significant cell viability reduction, cell cycle analysis was performed. Such analysis indicated that all three fractions could induce a G$_1$ cell cycle arrest (Figure 2), which was further confirmed by the down-regulation of CDK2 and CDK4 concomitant with the up-regulation of p21^{WAF1} and p27^{Kip1} (Figure 3). Thus, these results suggest that the cell viability reduction induced by these fractions is at least in part associated with a G$_1$ cell cycle arrest. In fact, the similar study using a different PL extract revealed a G$_1$ cell cycle arrest, accompanied by the same modulation of four cell cycle regulators mentioned above, in human leukemic cells [16]. However, separate studies also reported that other different PL extracts/compounds induced a S-phase cell cycle arrest in human hepatoma [25] and colorectal cancer cells [26]. Nevertheless, active compounds of PL with potential anticancer activity have been well characterized and those include hispolon, caffeic acicd, davallialactone, interfungins A, inoscavin A, several types of polysaccharides, proteoglycans etc. [6]. However, it has not been fully elucidated which active compound(s) would specifically induce a G$_1$ or S cell cycle arrest in what types of cancer cells. Although it is understandable that preparation of a pure form of each active compound would be a time-consuming and complex task, such studies are underway elsewhere. Meanwhile, the fact yet remains that different extracts or

compounds isolated from PL may vary with anticancer potency and mechanism as well as cancer specificity, suggesting their potential clinical implications.

Another interesting aspect of this study is that the up-regulation of p21^{WAF1} by PL-fractions (Figure 3) tempted us to explore their possible effects on the chromatin structure because p21^{WAF1} is associated with histone modifications [20]. In fact, activity of histone deacetylase (HDAC), one of the key regulators of histone acetylation [22], was *reduced* by three fractions, subsequently leading to hyperacetylation of specific histones [20] that can eventually up-regulate p21^{WAF1}. This p21^{WAF1} up-regulation would likely bring about a G$_1$ cell cycle arrest. Thus, PL-fractions may modify the chromatin structure or have some epigenetic effects, eventually reducing cell viability via a cell cycle arrest. Actually, this finding could be more significant in terms of possible epigenetic effects of PL-fractions. In particular, an additional study on specific inhibition of HDAC (blocking with siRNA) and activation of histone acetyltransferase (HAT) (as opposed to HDAC) by PL-fractions may further solidify our finding. Moreover, DNA methylation is also known as a major epigenetic control for gene transcription [27], and such hypermethylation of genes will then lead to the loss of biological functions of cancer cells including cell proliferation/viability. It is thus worthwhile performing such study to address if PL-fractions have any effects on the methylation status of DNA for further confirming their epigenetic effects.

Lastly, enzymatic assays on Csp-3 and Csp-9 showed that they were significantly ($p<0.03$) activated by these fractions, indicating induction of apoptosis [23,24]. Such activation of Csp-3/9 (leading to apoptosis) by some other PL compounds has also been reported in human leukemia [16] and colon cancer cells [28]. Thus, it is plausible that all three PL-fractions tested here could commonly activate Csp-3/9 and induce apoptosis in 5637 cells as well. In addition, other PL extracts/compounds have been well documented to induce apoptosis (indicated by other than Csp-3/9 activation) in several cancers, such as prostate [10,11], lung [12], colorectal [29,30], and melanoma [15]. Taken together, three distinct fractions of a single mushroom (PL) appear to have the diversified properties and could be useful in treating a variety of human malignancies.

5. Conclusions

This study demonstrates that three bioactive fractions of PL, namely PL-I, PL-II, and PL-III, have their anticancer activities on bladder cancer cells *in vitro*, although PL-III is the most potent fraction. The reduced cell viability through anticancer activity is accompanied by a G$_1$ cell cycle arrest, which is presumably induced by histone modifications. Ultimately, such a cell cycle arrest would result in apoptosis. Therefore, PL-fractions appear to be promising and may provide a safer and more effective therapeutic option for urothelial cell carcinoma as well as possibly other malignancies. While these findings are encouraging, further studies are warranted.

Abbreviations

UCC: urothelial cell carcinoma; TUR: transurethral resection; PL: Phellinus linteus; DMSO: dimethyl sulfoxide; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; CDK: cyclin-dependent kinase; Csp: caspase; HDAC: histone deacetylase; SD: standard deviation; ANOVA: one-way analysis of variance.

Conflict of Interest

The authors have no competing interests.

Acknowledgements

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