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Anticancer Activity of Maitake D-Fraction (PDF) against Three Highly Aggressive Cancer Cells

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Abstract

We have been studying anticancer effect of Maitake D-fraction (PDF), isolated from maitake mushroom, on various cancer cells in the past 24 years. PDF was highly effective on those cells, profoundly reducing their cell viability. However, we have not yet examined PDF on other cancer cells, particularly pancreatic, cervical, and small-cell lung cancer cells, which had been known to be dismal and deadly. Hence, we investigated if PDF alone or its combination with vitamin C (VC) might have a significant anticancer effect against those cancer cells. Pancreatic cancer AsPC-1, cervical cancer HT-3, and small-cell lung cancer H69AR cells, were treated with PDF or the combination of PDF and VC for 72 or 96 h and cell viability was determined to assess anticancer effect. The potential anticancer mechanism was also explored, focusing on glycolysis, chromatin structure, and apoptosis. A dose-dependent study showed that ≥30 μg/ml, ≥50 μg/ml, and ≥50 μg/ml of PDF led to the significant *reduction* in cell viability of AsPC-1, HT-3, and H69AR cells, respectively – the greater cell viability reduction is indicative of the greater anticancer effect. When these cancer cells were treated with the combination of PDF and VC with their ineffective/negligeable concentrations, their cell viability was more profoundly *reduced*, demonstrating a synergistic potentiation. Moreover, such a potentiation was primarily associated with increased oxidative stress (OXS), glycolysis inhibition, chromatin modifications, and ultimate apoptosis. In conclusion, the present study demonstrates anticancer effect of PDF and its synergistic potentiation with VC against AsPC-1, HT-3, and H69AR cells. Particularly, the enhancement of anticancer effect with the PDF/VC combination is rather extraordinary, accompanied by alterations in the essential cellular events. Therefore, PDF alone or its combination with VC may have some clinical implications in patients with various cancers.

Keywords

Maitake D-Fraction, PDF, Vitamin C, Anticancer, Pancreatic Cancer,

Cervical Cancer, Small-Cell Lung Cancer

1. Introduction

We have been studying to find the better therapeutic modality for urological cancers, such as prostate, bladder, and kidney cancers, using *natural products* as an *unconventional* approach. In particular, *PDF*, the extract of maitake mushroom (*Grifola frondosa*) [1], has been extensively studied for its anticancer effect against these cancer cells. Its bioactive component has also been identified as *β (beta)-glucan*, a protein-bound polysaccharide, with a molecular weight of ~1×10^6 Da [1]. This product is commercially available as a dietary supplement because its safety has been granted (exempt from a Phase 1 toxicology test) by the US Food and Drug Administration (FDA).

Our studies thus far showed that PDF itself had anticancer effect on these urological cancers, significantly reducing their cell viability [2]. Moreover, once PDF was combined with vitamin C (VC), anticancer effect was synergistically enhanced, leading to approximately 90% cell viability reduction in these cancer cells [2]. We then wondered if such profound anticancer effect could be specific to not only urological but also non-urological cancers. Hence, we were tempted to examine possible anticancer effect of PDF alone or the PDF/VC combination on three non-urological cancers, including pancreatic, cervical, and small-cell lung cancer cells.

Pancreatic cancer is one of the most aggressive and deadly cancers with the new cases of 66,000 and deaths of 52,000 (*i.e.*, ~80% of new cases) in 2024 [3]. Actually, the 5-year survival rate is merely 6%, due to early metastasis and chemotherapy (particularly with gemcitabine) resistance [4]. Although early detection and immediate surgical cancer excision are crucial, pancreatic cancer is mostly asymptomatic and only <20% of patients will receive an early diagnosis for viable treatments [4]. Due to the absence of specific/effective therapeutic options, novel and improved modalities are urgently demanded. For instance, it has been reported that several natural products, such as vegetables and fruits appeared to be effective on pancreatic cancer cases [5]. In fact, they have been shown to have anticancer activity with little side effects to cause/trigger secondary systemic problems [5].

Cervical cancer is the 4th common female cancer with the estimated new cases of 14,000 and deaths of 4,300 this year [3]. Interestingly, this cancer is often a result of being "infected" by some types of human papillomavirus (HPV) [6]. It is then believed that HPV is a critical factor required for the development of cervical cancer, so that the other risk factors can increase the cancer risk, but are not able to develop this cancer without HPV [7]. Although several therapeutic options, including surgery, chemotherapy, radiotherapy, vaccines, antibody-based therapies etc. [8] [9], the outcomes have not yet been properly assessed or uncertain. Nevertheless, it has been reported that "β-glucans" from various sources, such as yeast,

plant, bacterium, fungus etc., could be used to prevent/treat this cancer [10]. Hence, β-glucans may have the therapeutic potential of sole or adjuvant utility in the cervical cancer treatment.

Regarding lung cancer, it is the third leading cancer with the estimated new cases of 235,000 and deaths of 125,000 this year [3]. It is indeed a major disease with a significant economic burden in the US. Although extensive and intensive research has been conducted in decades, no breakthrough has ever been made yet. In fact, the current standard treatments include surgery, radiotherapy, chemotherapy, targeted therapy, immunotherapy etc. [11], but more effective modality needs to be urgently established. Meanwhile, to seek for a better therapeutic option, we have previously tested PDF on lung cancer A549 cells, representing the most common lung cancer (~85%) known as non-small cell lung cancer (NSCLC) [12]. Our study then showed the significant anticancer effect of PDF against A549 cells [13]. However, lung cancer also includes another lethal type classified as small-cell lung cancer (SCLC) with merely 15% incidence [14]. This lung cancer is yet highly aggressive and has the multi-drug resistance (MDR) nature with no effective treatments currently available, resulting in high mortality [14]. It then tempted us to examine if PDF or the PDF/VC combination would be capable of reducing cell viability in deadly SCLC cells.

Accordingly, we investigated anticancer effect of PDF or the PDF/VC combination on pancreatic, cervical, and small-cell lung cancer cells. To explore the anticancer mechanism, we also examined the possible involvement of oxidative stress (OXS), the status of glycolysis and chromatin structure, and induction of apoptosis. More details and interesting findings are described and discussed herein.

2. Materials and Methods

2.1. Cell Culture

Three cancer cell lines, pancreatic cancer AsPC-1, cervical cancer HT-3, and small-cell lung cancer H69AR cells, were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cells were essentially cultured in RPMI-1640 medium (Corning, Corning, NY), containing fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml), but additional reagents were also supplemented in the medium used in H69AR cells. They were incubated in a 37˚C - incubator, fed every 3 or 4 days, and passaged weekly. For experiments, HT-3 and H69AR cells were seeded at the initial cell density of 2×10^5 cells/ml in the 6-well plates or T-75 flasks, while AsPC-1 cells were seeded at 3×10^5 cells/ml. In general, both HT-3 and H69AR cells were then treated with PDF alone or PDF/VC combination for 72 h, while AsPC-1 cells were treated for 96 h. Those cells were subjected to either cell viability test (cultured in the 6-well plates) or cell harvest for biochemical analyses (cultured in the flasks).

2.2. MTT Assay (Cell Viability Test)

We define anticancer effect as the percent (%) of cancer cells that are still "alive"

following the treatments (with PDF or PDF/VC combination): the *higher* reduction in cell viability indicates the *greater* anticancer effect. At the end of treatment, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) reagent (1 mg/ml) was added to the 6-well plates containing three different cancer cells, followed by 3-h incubation at 37˚C. MTT reagent was discarded and 1 ml each of dimethyl sulfoxide was added to the plates to dissolve formazan precipitates (purple). The plates were then read at 595 nm on a microplate reader, and cell viability was expressed by the % of viable cells relative to the control (untreated) reading (100%).

2.3. Lipid Peroxidation (LPO) Assay

The severity of oxidative stress (OXS) induced with PDF alone or PDF/VC combination was assessed by LPO assay, measuring the amount of malondialdehyde (MDA) formed in the plasma membrane due to oxidative stress [15]—*the more MDA formed, the greater OXS.* The detailed procedures were described in the vendor's protocol (ABCAM, Waltham, MA). Briefly, three cancer cells exposed to PDF or PDF/VC combination for 3 or 6 h were lysed to obtain cell extracts. The reaction was then initiated by mixing cell extracts (of three different cells) with Thiobarbituric Acid (TBA) solution and incubated in a boiling water bath (~100˚C) for 1 h. Samples were read at A_{532} on a microplate reader, and the amount of MDA formed was determined by MDA standards and expressed by the fold increase relative to control (1).

2.4. Hexokinase (HK) Assay

Hexokinase (HK) activity was determined using HK Colorimetric Assay Kit (ABCAM, Waltham, MA) following the manufacturer's protocol. Cell lysates (20 μg per sample) obtained from three different cells and NADH standards were prepared in the same one 96-well plate and the reaction was started by the addition of reaction mixture (containing substrate). The plate was placed in a microplate reader and the absorbance (OD) changes with time were monitored at 450 nm for 20 min with 5-min intervals. All readings were calculated using references (NADH standards), and HK activity was expressed by the % of sample readings relative to the controls (100%).

2.5. Assay for Cellular ATP Level

The cellular ATP level was determined using ATP Colorimetric Assay Kit (ABCAM) following vendor's protocol. Three cancer cells ($2 - 3 \times 10^5$ cells/ml) cultured in the three separate 6-well plates were first lysed in ATP assay buffer, deproteinized with $HClO_4$, and neutralized with KOH. Cell lysates (50 μl per sample) obtained from three different cells and ATP standards were prepared in one 96-well plate and the reaction was started by the addition of reaction mixture. The plate was then incubated at room temperature for 30 min in the dark. Absorbance at 570 nm was read on a microplate reader and ATP contents in samples were calculated

by referring to the readings of ATP standards. The ATP level was then expressed by the % of ATP amounts in samples relative to the controls (100%).

2.6. Assays for DNA Methyltransferase (DNMT) and Histone Deacetylase (HDAC) Activities

DNMT and HDAC activities were determined essentially following the vender's protocol (EpiGentek, Farmingdale, NY). Three different control and agent-treated cells were harvested at 72/96 h and nuclear extracts were prepared using EpiQuik Nuclear Extraction Kit (EpiGentek). DNMT assay was performed on nuclear extracts using EpiQuik DNA Methyltransferase Activity/Inhibition Colorimetric Assay Kit (EpiGentek) following the given protocol. Similarly, HDAC assay was performed on nuclear extracts above using EpiQuik HDAC Activity/Inhibition Colorimetric Assay Kit (EpiGentek) as well. DNMT and HDAC activities were then calculated and expressed by the % relative to controls (100%).

2.7. Enzymatic Assays for Caspase-9 (Csp-9) and -3 (Csp-3)

Enzymatic activities of Csp-9 and Csp-3 were determined following the colorimetric method described in the manufacturer's protocol (ABCAM, Waltham, MA). Briefly, control or treated cells (~1×10^6 cells) were first lysed to obtain cell lysates. An equal amount (100 μg) of cell lysates was prepared in the 96-well plate and incubated with the reaction buffer containing the substrates, either LEHD-pNA (for Csp-9) or DEVD-pNA (for Csp-3), at 37˚C for 2 h. The optical density (OD) of the reaction mixture was then read at 405 nm in a microplate reader. Following calculations, Csp-9/3 activities were expressed by the fold increase of treated samples compared with untreated control sample (1).

2.8. Statistical Analysis

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

3. Results

3.1. Anticancer Effect of PDF alone or PDF/VC Combination on Three Cancer Cells

A dose-dependent study was performed on AsPC-1, HT-3, and H69AR cells by treating them with varying concentrations (0 - 500 μg/ml) of PDF alone or combinations of PDF and VC with specific concentrations for 72 or 96 h, and cell viability was determined by MTT assay. Both HT-3 and H69AR cells were cultured for 72 h, while AsPC-1 cells were for 96 h because they grew relatively *slower* than other cells and needed an extra day to reach confluency. The results showed that ≥30 μg/ml, ≥50 μg/ml, and ≥50 μg/ml of PDF led to the significant reduction in cell viability of AsPC-1, HT-3, and H69AR cells, respectively (**Figures 1(a)-(c)**). Human bladder

cancer T24 cells were also included here as the reference control, which showed with the effective concentrations of ≥350 μg/ml (Figure 1(d)).

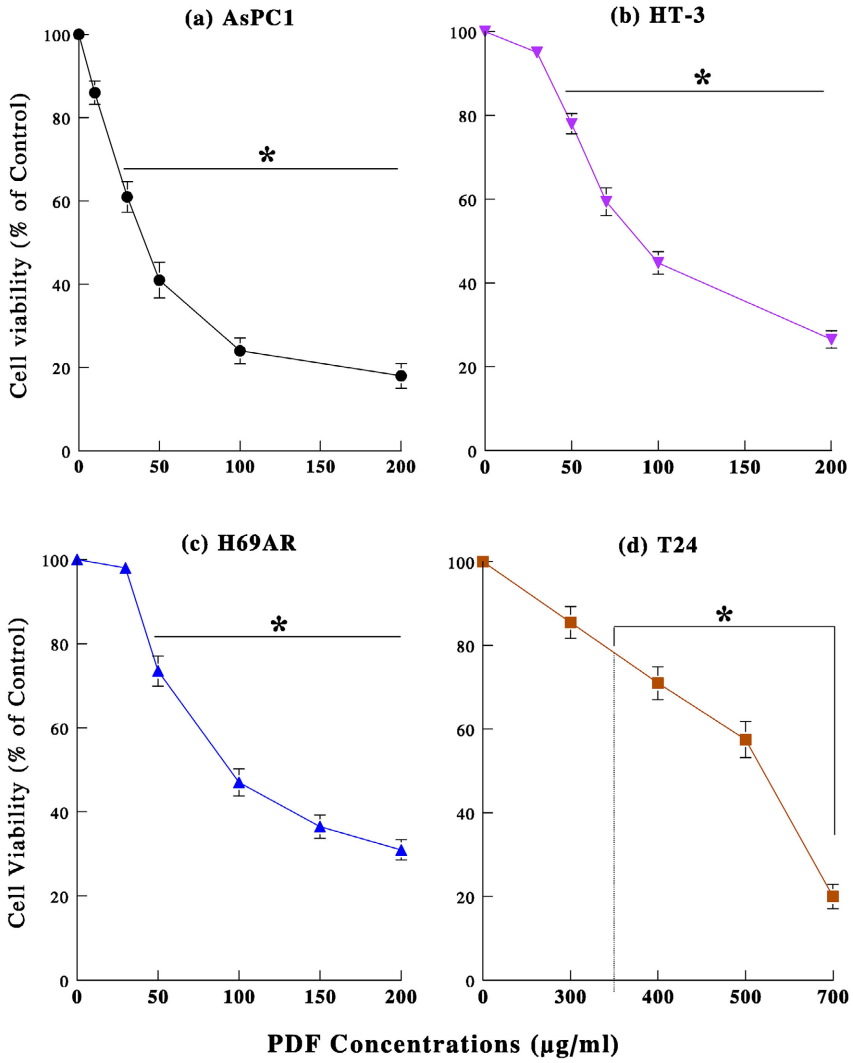

Figure 1. Dose-dependent effect of PDF. AsPC-1 (a), HT-3 (b), H69AR (c), and T24 (d) cells were treated with given concentrations of PDF, and cell viability was determined in 72 or 96 h. All data are mean ± SD (standard deviation) from three separate experiments. Cell viability was then expressed by the % of viable cells in treated cells relative to control (100%) (*$p < 0.05$ compared with control).

3.2. Synergistic Potentiation of PDF with Vitamin C (VC)

As it has been shown that bioactivity of β-glucan (active component of PDF) could be potentiated with VC [16], this possibility was tested next on three cancer cells. The *ineffective* concentrations of VC used were determined earlier in the pilot study, and the combinations of *ineffective* PDF and VC were examined as follows: the combination of PDF (10 μg/ml) and VC (300 μM) in AsPC-1, PDF (30 μg/ml) and VC (300 μM) in HT-3, PDF (30 μg/ml) and VC (300 μM) in H69AR, and PDF (300 μg/ml) and VC (100 μM) in T24 cells included as a positive control.

HT-3, H69AR, and T24 cells at 72 h or AsPC-1 cells at 96 h were subjected to cell viability test. Such results showed that cell viability was significantly reduced to ~45%, ~23%, ~34%, and ~10% with the respective PDF/VC combination in AsPC-1, HT-3, H69AR, and T24 cells, respectively (Figures 2(a)-(d)). Thus, all cancer cells tested here show the profound reduction in their cell viability with the PDF/VC combination, which is far greater than with PDF alone. Since this is more likely due to a *synergistic* potentiation of PDF with VC, these specific combinations here were then used in the rest of our study unless otherwise indicated.

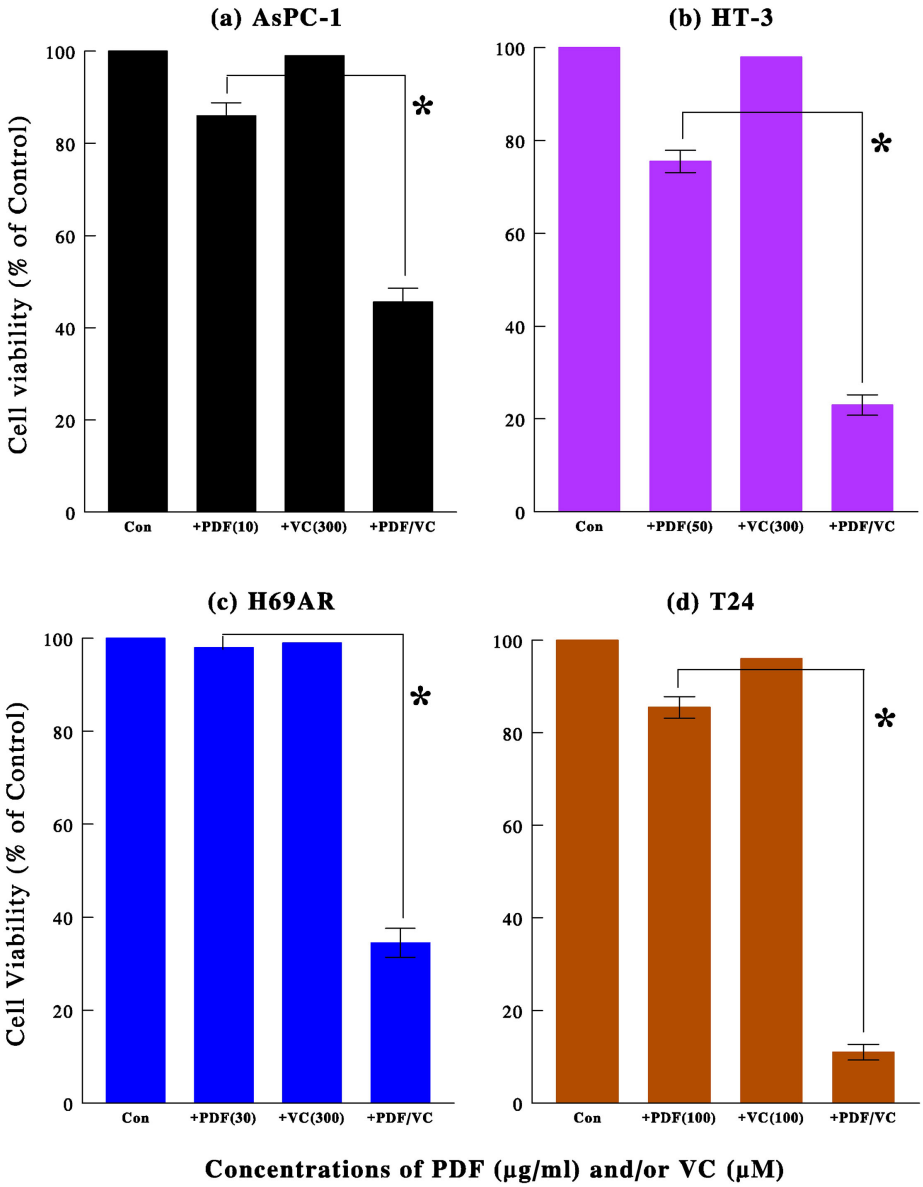

Figure 2. Synergistic potentiation of anticancer effect with PDF/VC combination. AsPC-1 (a), HT-3 (b), H69AR (c), or T24 (d) cells were treated with PDF alone, VC alone, or PDF/VC combination. They were subjected to MTT assay at 72/96 h and all data were mean ± SD from three independent experiments. Cell viability was expressed by the % relative to control (100%) (*$p < 0.05$ compared PDF alone with PDF/VC combination).

3.3. Induction of Oxidative Stress (OXS) with PDF alone or PDF/VC Combination

To explore the anticancer mechanism of PDF alone or PDF/VC combination, we first examined if they had *prooxidant* activity to induce oxidative stress (OXS), enhancing anticancer activity. AsPC-1, HT-3, and H69AR cells were briefly exposed to PDF or PDF/VC combination for 3 or 6 h and subjected to lipid peroxidation (LPO) assay to assess the severity of OXS induced. The results showed that the severity/level (measured by amount of MDA formed) of OXS with PDF alone appeared to be higher but was not significant ($p = 0.05$) in three cancer cells (Figure 2(a)). However, the severity of OXS was >5-fold *greater* with PDF/VC combination (than those of respective control cells) (Figure 2(b)). These results suggest that some OXS could be induced with PDF alone but may not be significantly linked to the cell viability reduction (due to cell death). In contrast, PDF/VC combination appears to induce *greater* OXS that would subsequently result in cell damage and cell death. Thus, PDF/VC combination has a *prooxidant* effect, which may severely exert OXS on cells, primarily accounting for the profound reduction in cell viability in all three cancer cells.

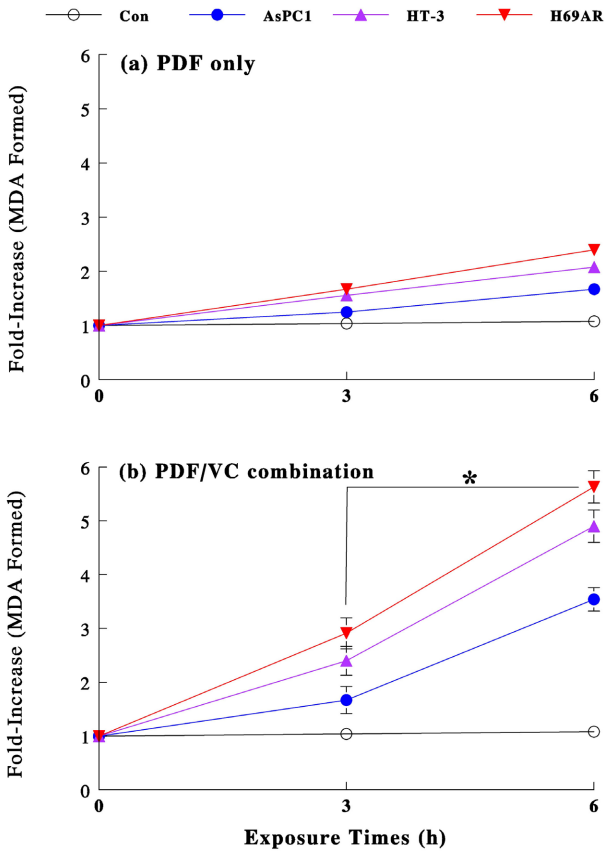

Figure 3. Induction of oxidative stress (OXS). AsPC-1, HT-3, or H69AR cells briefly treated with PDF alone or PDF/VC combination for 3 or 6 h were subjected to LPO assay. The amount of MDA formed (indicator of OXS) was calculated (based on MDA standards) from three separate experiments and expressed by the fold increase relative to control (1) (*$p < 0.05$ compared with control).

3.4. Inhibition of Glycolysis

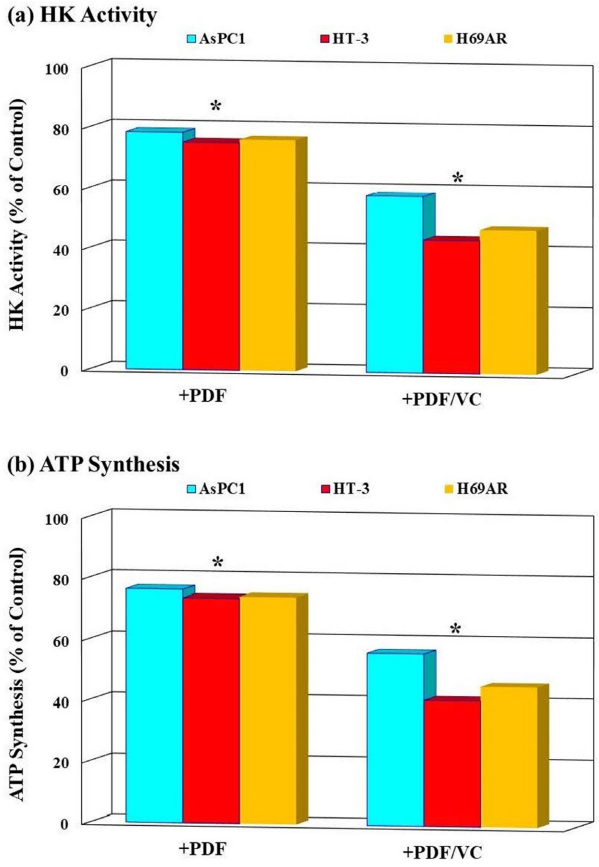

Figure 4. Inhibition of glycolysis. AsPC-1, HT-3, or H69AR cells treated with PDF alone or PDF/VC combination for 72/96 h were subjected to hexokinase (HK) assay (a) or assay for ATP synthesis (b) All data are mean ± SD from three independent experiments, and HK activity and ATP synthesis in treated cells are expressed by the % relative to control (100%) (*$p < 0.05$ compared with control). No error bars (SD values) are shown here.

There was a possibility that OXS could disrupt/inhibit glycolysis, the vital metabolic process required for cell proliferation and survival [17], and the disruption of glycolysis has been reported to inhibit the growth of cervical cancer cells [18]. We then examined the status of glycolysis in three cancer cells treated with PDF alone or PDF/VC combination by assessing the two parameters, hexokinase (HK) activity and ATP synthesis. HK is one of key glycolytic enzymes, which is involved in the irreversible committed step in glycolysis [19], and its inactivation/inhibition would disrupt glycolysis, leading to the reduction in ATP synthesis. AsPC-1, HT-3, and H69AR cells were treated with PDF alone or PDF/VC combination for 72/96 h, and they were first subjected to HK assay. The results showed that HK activities in three cancer cells were lost by >20% and >50% with PDF and PDF/VC combination, respectively (**Figure 4(a)**). ATP assay revealed that the amount of ATP synthesized was also significantly reduced with treatments (**Figure 4(b)**). Such inactivation of HK and the reduction in ATP synthesis apparently indicates the inhibition or incompletion of the glycolytic pathway. Therefore, it is plausible

that PDF alone or PDF/VC combination substantially inhibits glycolysis, resulting in the growth cessation and/or cell death.

3.5. Induction of Chromatin Modifications

OXS has also been shown to cause DNA damage or chromatin modifications (DNA methylation, histone acetylation etc.), which could result in a number of cellular changes including cell death [20]. We examined a possible impact of OXS on the chromatin structure, focusing on two key epigenetic regulators, DNA methyltransferase (DNMT) and histone deacetylase (HDAC) [20] [21]. Three cancer cells treated with PDF alone or PDF/VC combination for 72/96 h were assayed for activities of DNMT and HDAC. Such assays showed that PDF alone led to a *slightly* significant ($p \leq 0.05$) loss (>20%) in activities of both DNMT and HDAC in all cells (**Figure 5(a)**), suggesting some alterations in chromatin structure. In contrast, DNMT/HDAC activities were *significantly* ($p < 0.05$) lost (>40%) with PDF/VC combination (**Figure 5(b)**), indicating substantial chromatin modifications. Thus, PDF alone and PDF/VC combination would induce DNA modifications, presumably resulting in cell death. This may further account for the cell viability reduction.

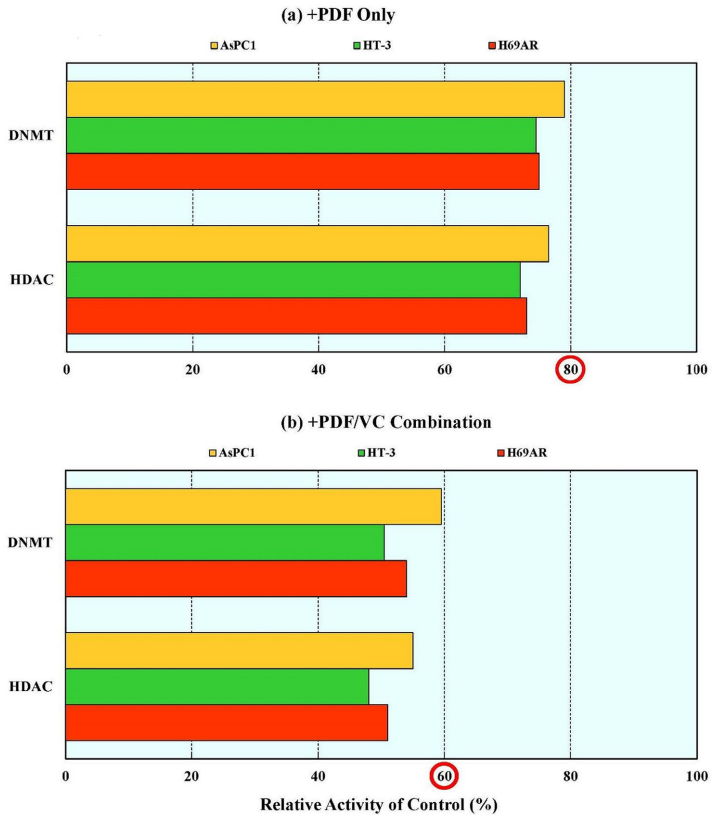

Figure 5. Induction of chromatin modifications. AsPC-1, HT-3, or H69AR cells treated with PDF alone or PDF/VC combination for 72/96 h were assayed for activities of DNMT and HDAC. All activities were calculated from three separate experiments and expressed by the % relative to control (100%). Both DNMT and HDAC activities were significantly lost more in PDF/VC-treated cells than those treated only with PDF (*$p < 0.05$ compared with control). No error bars (SD values) are shown.

3.6. Induction of Apoptosis

Lastly, we examined the fate of cells treated with PDF alone or PDF/VC combination—whether they would ultimately undergo "apoptosis". It is a unique form of cell death, regulating (cancer) cell survival [22], as it eliminates cancerous cells through atrophy of cells and execution of the cell suicide pathway [23].

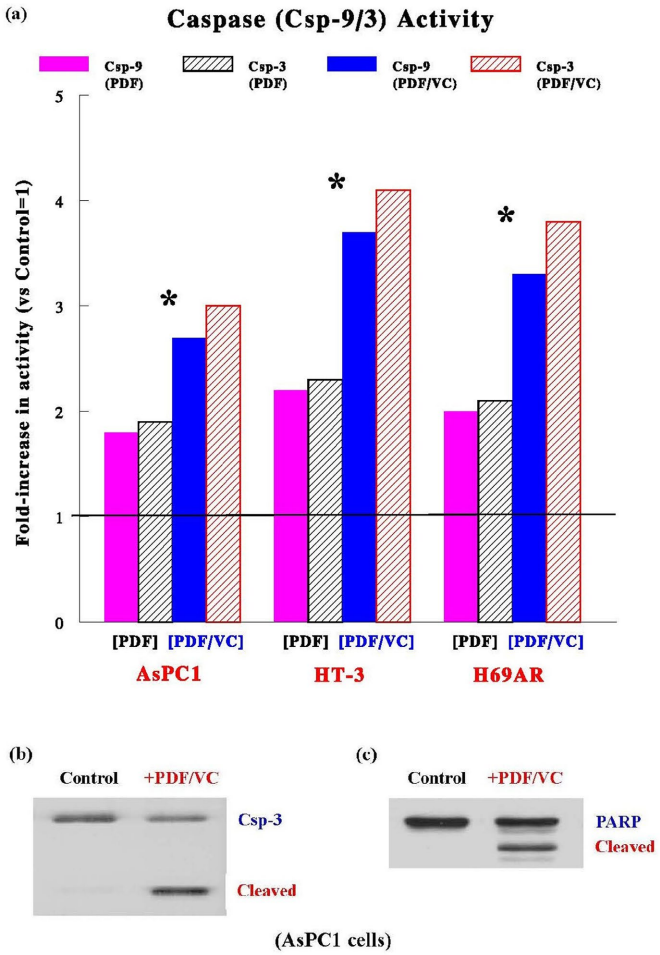

Figure 6. Induction of apoptosis. (a) Three cancer cells treated with PDF alone or PDF/VC combination for 72/96 h were subjected to enzymatic assays for Csp-9/3 activities. All activities were calculated from three independent experiments and expressed by the fold increase relative to control (1) (*$p < 0.05$ compared with control). Additionally, activation of Csp-3 is qualitatively shown as its cleavage (b) as well as activation PARP resulted from its cleavage (c) in AsPC1 cells using Western blot analysis.

We analyzed enzymatic activities of two key regulators involved in apoptosis – specifically, caspase 9 & 3 (Csp-9 & Csp-3) [24] [25]. AsPC-1, HT-3, and H69AR cells treated with PDF or PDF/VC combination for 72/96 h were subjected to enzymatic assays for Csp-9/3. Such results revealed that activities of Csp-9/3 in all three cells were significantly ($p < 0.05$) *higher* with PDF or PDF/VC combination than that in control (**Figure 6(a)**). Such activation of Csp-9/3 has been known to *promote apoptosis* [24] [25]. In addition to this quantitative assay, activation of

Csp-3 was also qualitatively demonstrated by release of its active cleaved form with PDF/VC combination in AsPC-1 cells, revealed by Western blot (**Figure 6(b)**). Moreover, another key apoptotic factor, poly (ADP-ribose) polymerase (PARP) [26], was found to be activated with PDF/VC treatment, evidenced by detection of its cleaved band on Western blot (**Figure 6(c)**). Such activation of PARP is also known to induce/promote apoptosis [27]. Taking together, these results further confirm the induction of apoptosis. Therefore, all cancer cells treated with PDF alone or PDF/VC combination would ultimately undergo apoptosis.

4. Discussion

The three cancers, pancreatic (AsPC-1), cervical (HT-3), and small-cell lung (H69AR) cancers, are highly aggressive cancers, and there are few effective therapeutic modalities currently available. To find the better or improved treatments, we investigated if Maitake D-fraction, PDF, might have anticancer effect against these cancer cells and particularly when it is combined with VC. Actually, a wide variety of cancer cells, including breast, lung (non-small-cell), stomach, colorectal, liver, brain, leukemia etc., have all resulted in a significant cell viability reduction with PDF alone or PDF/VC combination [2]. Hence, we examined anticancer effect of PDF and its combination with VC on additional three fatal cancer cells this time.

First of all, all three cancer cells resulted in the significant cell viability reduction with ≤50 μg/ml of sole PDF (**Figure 1**), demonstrating its anticancer effect. Such a cell viability reduction became further greater when PDF was combined with VC (i.e., PDF/VC combination) (Figure 2). Since these concentrations of PDF and VC used were *ineffective* or negligible by themselves, the resulting profound reduction in cell viability (55% - 77%) is most likely attributed to a *synergistic* potentiation. Hence, it is plausible and promising that the relatively *low* concentrations of PDF and VC are required to be effective on these cancers.

In the meantime, we attempted to understand *how* anticancer effect of PDF/VC combination was greatly enhanced or what the underlying mechanism would be. The involvement of VC insinuated us its possible *prooxidant* activity. Although VC is generally and widely known as an antioxidant, it has a dual activity acting as an *antioxidant* or a *prooxidant* [28]. It has been shown that VC can act as a prooxidant (facilitating OXS) with specific elements or under certain microenvironment [29]. Hence, we examined if the severity of OXS would intensify when PDF was combined with VC. While PDF alone slightly increased OXS (compared to that in controls), the PDF/VC combination exceedingly elevated OXS over 5-fold greater than controls (**Figure 3**). This suggests that such intensified OXS will severely and extensively attack and harm/kill those cancer cells, principally accounting for the profound cell viability reduction.

Nevertheless, exactly how the combination of PDF and VC will induce OXS yet remains unknown. Active component of PDF is *β-glucan*, which is the well-characterized, unique protein-bound polysaccharide [1], but the chemical reaction between this *β*-glucan and VC to generate OXS has not been scrutinized and fully

understood. It is certainly interesting and important to understand and elucidate induction of OXS, but it is also beyond the scope of our current study at this point.

We then explored how such elevated OXS might have adverse impacts on the cellular and nuclear levels. Since glycolysis is a crucial metabolic process for cell proliferation and survival [17], possible effects of OXS on glycolytic parameters were examined. We found that HK, the key rate-determining enzyme in glycolysis [19], was significantly *inactivated* and ATP synthesis was also subsequently *reduced* with the PDF/VC combination (**Figure 4**). These results thus suggest that glycolysis is extensively inhibited, eventually leading to the significant reduction in cell viability.

In addition, OXS has been shown to affect not only the cellular level (e.g., glycolysis) but also the nuclear level involving DNA and chromatin [20] [21]. The chromatin structure was then assessed by activities of the two epigenetic regulators, DNMT and HDAC. The results showed that activities of both regulators were significantly inactivated/lost with the PDF/VC combination (**Figure 5**), indicating chromatin modifications [21].

Taken together, OXS appears to adversely affect both the cellular and nuclear levels, further accounting for such an extensive cell viability reduction induced with the PDF/VC combination. Those treated cells were found to actually undergo apoptosis, leading to widespread cell death. Therefore, the PDF/VC combination is indeed capable of ultimately inducing apoptosis in all three cancer cells.

Speaking of apoptosis, we believe that *induction of apoptosis* has a clinical relevance because of how cancer cells will die [23]. Apoptosis following the highly organized biochemical death pathway will result in few secondary inflammations or "side effects" unlike necrosis involving chaotic abrupt cell death with severe side effects (e.g., chemotherapy). Hence, any agents such as PDF, capable of inducing apoptosis in cancer cells, would be safer and more effective with few side effects when they are given to cancer patients.

Lastly, it is important to mention the physiologically achievable concentrations of PDF and VC, although the relatively low concentrations seem to be required. Nevertheless, no physiologically achievable concentration of PDF has yet been established at present. Hence, animal study is crucial and required to determine such a concentration. In the meantime, a stock PDF (sold commercially) has a 30 mg/ml concentration, which is 600 times more concentrated than 50 μg/ml used in this study. A daily oral intake of 60 mg PDF (2 ml) is currently recommended/used in the volunteer-based clinical study without any adverse effects. However, exactly what physiological concentration will be achieved is uncertain. Regarding the physiologically achievable concentration of VC, it has been shown in a clinical trial that the intravenous (IV) infusion of VC in cancer patients safely reached plasma concentrations over 10 mM [30]. This suggests that "300 μM" VC can be physiologically achievable in patients/subjects. Therefore, both PDF and VC appear to be the practical and promising agents for treatment of these three aggressive cancer cells.

5. Conclusion

The present study demonstrates that the bioactive extract of maitake mushroom, PDF, has anticancer effect on three cancer cells tested, AsPC-1, HT-3, and H69AR cells. When PDF was combined with vitamin C (VC), anticancer activity was synergistically potentiated, resulting in the significant cell viability reduction in all three cancer cells. Such a reduction induced with PDF/VC combination was primarily attributed to oxidative stress (OXS), leading to the glycolysis inhibition, chromatin modifications, and ultimate apoptosis. Therefore, PDF could be considered as a safer and more effective alternative with few side effects, which might be used in patients with pancreatic, cervical, or small-cell lung cancers.

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Conflicts of Interest

All authors declare no conflicts of interest regarding the publication of this paper.

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