Potentiation of Anticancer Effect by Combination of Mushroom Extract and Grapeseed Extract on Human Bladder Cancer Cells

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ABSTRACT

Background: Although bladder cancer is the second-leading urologic cancer next to prostate cancer in the United States, few effective and satisfactory therapeutic options have yet been established. As we have been searching for certain natural remedies with anticancer activity, we came across the two natural products, namely mushroom extract (PL) and grapeseed extract (GSP). Accordingly, we investigated if the combination of these two extracts could further potentiate anticancer effect on bladder cancer cells.

Materials and Methods: Human bladder cancer T24 cells were separately treated with PL, GSP, or their combination, and cell viability was determined by MTT assay. To explore the anticancer mechanism, severity of oxidative stress was assessed by lipid peroxidation (LPO) assay, activities of epigenetic regulators were determined by enzymatic assays, and apoptotic regulators were examined by Western blot analysis.

Results: PL and GSP alone led to a ~20% and ~25% cell viability reduction at 20 µg/ml and 25 µg/ml, respectively. When the same concentrations of PL and GSP were then combined, cell viability was drastically reduced to merely ~15% (~85% reduction) in a synergistic manner. Concomitantly, the PL/GSP combination led to a ~3-fold increase in oxidative stress, assessed by LPO assay. Activities of DNA methyltransferase and histone deacetylase were significantly lost by ~50% and 55% with the PL/GSP combination, respectively. Moreover, Western blot analysis revealed that bcl-2 (anti-apoptotic regulator) was down-regulated while Bax (pro-apoptotic regulator) was up-regulated with the combination, implying induction of apoptosis.

Conclusions: This study shows that the combination of PL and GSP can synergistically induce the significant reduction (~85%) in T24 cell viability, demonstrating an extensive potentiation of anticancer effect. Such a profound effect is accompanied by increased oxidative stress, chromatin modifications, and ultimate apoptosis. Therefore, the PL/GSP combination may offer a more effective and safer regimen for bladder cancer.

Keywords
Anticancer, Bladder cancer, Oxidative stress, Phellinus linteus, Proanthocyanidin.

Introduction
Bladder cancer is the second most common urologic malignancy next to prostate cancer in the United States [1]. The majority of bladder cancers present as superficial (80%) with 15% presenting as invasive cancer and 5% as metastatic disease [2]. Currently, urothelial cell carcinoma (UCC) is the most prevalent primary bladder tumor: 84,000 new cases and 17,000 deaths are estimated this year (2021) [1]. Although endoscopic transurethral resection (TUR) is often performed as a primary therapy, 60%-70% of patients will recur in 5 years and ~25% will progress to muscle invasive disease [3]. Thus, the immediate therapeutic aim is to prevent multiple recurrences and progression to a more advanced, invasive disease.
Besides chemotherapy, intravesical administration of bacillus Calmette-Guerin (BCG), an attenuated strain of *Mycobacterium bovis*, is currently the most effective immunotherapy available for high-grade and recurrent superficial bladder cancer and carcinoma in situ (CIS) [4]. That said, side effects of BCG therapy are common: cystitis occurs in 90% of patients and hematuria in ~30% of patients, and other potential adverse effects (fever, allergic reactions, sepsis etc.) cannot be yet ruled out. These disadvantages thus limit its use in clinical practice, demanding a safer, more effective treatment modality with fewer side effects.

The medicinal aspects of various natural products/agents have received more public attention [5]. 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 [6]. To develop it for anticancer therapeutics, scientific and medical studies of PL have been intensively conducted. Those studies revealed that PL had antitumor, immunomodulatory, anti-angiogenic, and antioxidant properties [7-10].

In fact, its antitumor or anticancer activity has 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 [11-17]. It is thus plausible that such potential effects of PL may offer alternative therapeutic option for bladder cancer as well.

In addition, proanthocyanidins are naturally occurring plant polyphenolic bioflavonoids in fruits, vegetables, nuts, seeds, flowers and bark [18]. They are known as natural antioxidants, having biological, pharmacological and chemoprotective properties against oxidative stress or harmful free radicals [18-20]. For example, hydrogen peroxide-induced oxidative stress was significantly reduced by proanthocyanidins, protecting cultured macrophage and neuroactive PC-12 cells from lethal free radical attack [20]. They also demonstrated antibacterial, antiviral, anti-inflammatory and vasodilatory actions [20].

Particularly, a unique grape seed proanthocyanidin (GSP) has been extensively characterized: it is a standardized water-ethanol extract from red grape seeds, consisting of oligomeric proanthocyanidins as the major active components in 54% dimeric, 13% trimeric, 7% tetramer, and 6% monomeric forms and also other flavonoids [19]. This GSP has been shown to demonstrate its anticancer (cytotoxic) effect on several malignancies including breast, lung and gastric cancers in vitro [21].

Accordingly, we investigated whether PL, GSP or their combination might have the better anticancer effect on human bladder cancer cells in vitro and also explored such an anticancer mechanism for obtaining a better understanding.

### Materials and Methods

#### Cell Culture

The human bladder cancer T24 cells, derived from a patient with UCC, were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). For experiments, T24 cells were seeded in 6-well plates (2 ml/well) or T-75 flasks (10 ml) at the initial cell density of 1 x 10⁵ cells/ml and were cultured with *Phellinus linteus* extract (PL; Mushroom Wisdom, Inc., East Rutherford, NJ), grapeseed proanthocyanidin (GSP; Dry Creek Nutrition, Inc., Modesto, CA) or their combination. Cell viability was then determined at specified times following MTT assay described below.

#### MTT Assay

Anticancer effect can be assessed by cell viability where the % of viable cell number in treated-cells is relative to that in control cells (100%) – the greater reduction in cell viability is indicative of the greater anticancer activity. Cell viability was then determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay following the vendor’s protocol (Sigma-Aldrich, St. Louis, MO). After cells were treated with given agents in the 6-well plate for specified times, MTT reagent (1 mg/ml) was added to each well in the plate that was then incubated for 3 h in an incubator. Dimethyl sulfoxide (DMSO) was added to the plate to dissolve formazan precipitates (purple) and absorbance of samples was read in a microplate reader. Cell viability was then expressed by the % of sample readings (OD) relative to the control reading (100%).

#### Lipid Peroxidation (LPO) Assay

The severity of oxidative stress was assessed by the LPO assay measuring the formation of malondialdehyde (MDA), an end product from peroxidation of polyunsaturated fatty acids in the plasma membrane [22]. The amount of MDA formed indicates the severity of oxidative stress: the more MDA formed, the greater oxidative stress. The detailed procedures were described in the vendor’s protocol (ABCAM, Cambridge, MA), and the amounts of MDA formed were measured by µM and converted to arbitrary values relative to controls (1.0).

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

Assays for DNMT and HDAC were performed essentially following the vendor’s protocol (EpiGentek, Farmingdale, NY). Control and agent-treated cells for 72 h were harvested and nuclear extracts were prepared using EpiQuik Nuclear Extraction Kit (EpiGentek). DNMT assay was performed on nuclear extracts using EpiQuik DNA Methyl transferase 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 percentage (%) relative to controls (100%).
**Western Blot Analysis**

Briefly, an equal number of proteins (10 µg) obtained from control and agent-treated cell lysates was resolved by 10% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and transferred to a nitrocellulose membrane (blot), which was incubated with 5% milk overnight at 4°C. After discarding milk and rinsing the blot with a washing solution, the blot was first incubated for 90 min with the primary antibodies against bcl-2, Bax or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with the appropriate secondary antibody conjugates for 30 min. After discarding antibodies and rinsing the blot, the specific immunoreactive proteins (bcl-2, Bax, and β-actin) were then detected by chemiluminescence (Kirkegaard and Perry Laboratories, Gaithersburg, MD) on an X-ray film (autoradiograph).

**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.

**Results**

**Effects of PL and GSP on T24 Cell Viability**

To assess anticancer effect of PL and GSP, T24 cells were separately cultured with the varying concentrations of PL (0-150 µg/ml) or GSP (0-100 µg/ml) and cell viability was determined at 72 h. Such studies showed that PL at 20 µg/ml led to a ~20% cell viability reduction, which further declined to >65% with ≥100 µg/ml (Figure 1A). Similarly, 25 µg/ml GSP induced a ~25% cell viability reduction and a severe >80% viability reduction was seen with ≥50 µg/ml (Figure 1B). These results thus show that PL and GSP are capable of significantly reducing T24 cell viability, especially at their high concentrations (≥100 µg/ml with PL and ≥50 µg/ml with GSP).

**Synergistic Potentiation of Anticancer Effect with Combination of PL and GSP**

Next, we examined if the combination of PL and GSP might improve anticancer effect to further reduce T24 cell viability. Especially, it would be rather practical (for clinical use) if the relatively low concentrations of both PL and GSP could potentiate such anticancer effect. Cells were treated with 20 µg/ml PL (with a ~20% viability reduction) alone, 25 µg/ml GSP (with a ~25% viability reduction) alone, or their combination and then cell viability was determined at 72 h. While the ~20% and ~25% cell viability reduction with PL and GSP, respectively, were seen as expected, the PL/GSP combination resulted in a drastic 85% viability reduction (Figure 2). As such a reduction is far greater than an additive effect of two samples, it is considered to be a synergistic potentiation. Thus, the relatively low concentrations of PL and GSP with low/weak anticancer activity could become highly potent once combined, leading to a profound cell viability reduction.

**Increase in Oxidative Stress with PL/GSP Combination**

To understand how the PL/GSP combination can induce such a severe impact on cell viability (i.e. the anticancer mechanism), we then examined if oxidative stress (generation of reactive oxygen species) might play a certain role in this incidence. Actually, this possibility was supported by our observation where dead cells or dying cells exhibited unique morphological changes, such as cell detachment and cell blebbing, indicating possible exertion of oxidative stress on the cells [23]. T24 cells were treated with PL (20 µg/ml) alone, GSP (25 µg/ml) alone, or their combination for 6 h and the severity of oxidative stress was assessed by LPO assay. It is commonly known that oxidative stress generally takes place at the early stage/time [24] and 6 h was believed to be sufficient. LPO assay revealed that the marginal 1.3-fold and 1.5-fold increases in oxidative stress were seen with PL and GSP alone, respectively (Figure 3). In contrast, the PL/GSP combination led to a ~3-fold increase in oxidative stress, indicating exertion of severe oxidative stress (Figure 3). Thus, it is rather plausible that the PL/GSP-induced cell viability reduction could be primarily due to cell death (through oxidative stress).

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**Figure 1:** Effects of PL or GSP on T24 cell viability. Cells were cultured with the varying concentrations of either PL (0-150 µg/ml) or GSP (0-100 µg/ml), and cell viability of PL-treated (A) or GSP-treated (B) cells were determined at 72 h. Cell viability was assessed by the % of viable cell numbers relative to that in control cells (100%). All data represent mean ± SD (standard deviation) from three independent experiments (*p <0.05; **p <0.01).
Figure 2: Effects of combination of PL and GSP on cell viability. Cells were treated with PL (20 µg/ml), GSP (25 µg/ml), or their combination for 72 h, and cell viability was assessed. The data are mean ± SD from three separate experiments (*p < 0.03).

Figure 3: Assessment of severity of oxidative stress. After cells were treated with PL (20 µg/ml), GSP (25 µg/ml), or their combination for 6 h, the amount of MDA formed, which directly indicates severity of oxidative stress, was determined by LPO assay. Severity of oxidative stress was calculated and expressed by the fold increase relative to control cells (1). All data are mean ± SD from three independent experiments (*p < 0.05).
Possible Chromatin Modifications Induced by PL/GSP Combination

Oxidative stress has 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 [25]. We examined a possible impact of oxidative stress on the chromatin structure, focusing on two key epigenetic regulators, DNA methyltransferase (DNMT) and histone deacetylase (HDAC) [25,26]. Cells were treated with PL, GSP, or PL/GSP combination and activities of DNMT and HDAC were assessed at 72 h. The results showed that DNMT activity was slightly lost by ~11% and ~14% with PL and GSP, respectively, whereas DNMT was significantly inactivated by ~50% (~50% activity loss) with the PL/GSP combination (Figure 4A). Similarly, HDAC activity was lost by ~13% and ~16% with PL and GSP, respectively, but the PL/GSP combination led to a ~55% activity loss (Figure 4B). This significant inactivation of DNMT and HDAC with the PL/GSP combination represents chromatin modifications that can ultimately lead to cell death. Thus, such epigenetic changes may also in part account for the PL/GSP-induced cell viability reduction.

Figure 4: Chromatin modifications induced by oxidative stress. Cells treated with PL, GSP, or their combination for 72 h were assayed for activities of two epigenetic regulators, DNMT and HDAC. Such activities were calculated and expressed by the % relative to control (100%). All data are mean ± SD from three separate experiments (*p <0.05).

Induction of Apoptosis by PL/GSP Combination

Now, the last question was whether PL/GSP-induced cell death would be attributed to apoptosis (programmed cell death). Cells were treated with PL, GSP, or the PL/GSP combination for 72 h and two key apoptotic regulators, bcl-2 and Bax, were analyzed using Western blots. Such analysis revealed that expression of bcl-2 was reduced or down-regulated while that of Bax was enhanced or up-regulated with the PL/GSP combination (Figure 5). No such changes in bcl-2 and Bax with PL or GSP alone were observed, yet remaining the same expressions as those in control cells (data not shown). As bcl-2 is an anti-apoptotic regulator while Bax is a pro-apoptotic regulator [27], the down-regulation of bcl-2 and the up-regulation of Bax seen here indicates induction of apoptosis. Thus, PL/GSP-induced cell death more likely follows the apoptotic pathway.

Figure 5: Induction of apoptosis. Cells cultured with PL, GSP, or their combination for 72 h were subjected to Western blots to analyze the expressions of bcl-2 and Bax. Autoradiographs of their protein expressions in control and PL/GSP-treated cells are shown. β-actin is also included as a loading control. Note: those expressions in PL- or GSP-treated cells are not shown here.

Discussion

Due to the ineffective therapeutic options and unsatisfactory outcomes, bladder cancer patients and their families are desperately seeking for a more effective therapeutic modality. We have been exploring an alternative way to improve such a therapeutic efficacy and performed the study using PL, the bioactive mushroom extract, and GSP, the grapeseed extract, on human bladder cancer T24 cells. As both PL and GSP are the natural products, they have few side effects and are believed to have clinical implications on treatment of various cancers as well [7,21].

Our study showed that PL and GSP alone could induce a significant reduction in T24 cell viability at their higher concentrations (Figure 1). However, for a potential clinical use, we examined if the lower concentrations of these extracts could become more effective or potentiate their anticancer effects when they were combined. This study then demonstrated that the combination of relatively low concentrations of PL (20 µg/ml) and GSP (25 µg/ml) led to a profound 85% cell viability reduction, compared to a ~20% and ~25% viability reduction induced with individual
PL and GSP, respectively (Figure 2). This drastic cell viability reduction with the PL/GSP combination is far beyond an additive effect and indicative of a synergistic potentiation. Now, it was important to understand how such a combination was capable of further enhancing anticancer effect.

To explore the underlying anticancer mechanism of the PL/GSP combination, we first examined whether oxidative stress might play a significant role because we observed characteristic morphological changes likely due to oxidative stress. In particular, cell detachment and cell blebbing induced by the PL/GSP combination were the typical appearances seen under oxidative stress [23]. The severity of oxidative stress assessed by LPO assay showed that a significant 3-fold increase in oxidative stress (compared to control cells) was attained with the PL/GSP combination, whereas only the marginal increases were seen with individual PL and GSP (Figure 3). This clearly indicates that severe oxidative stress was exerted on T24 cells, feasibly leading to extensive cell death and the cell viability reduction. Therefore, PL/GSP-induced oxidative stress appears to be severe enough to induce adverse cellular alterations, resulting in the profound cell viability reduction.

It was then tempting to further investigate possible (adverse) effects of oxidative stress particularly on chromatin. It has been well known that oxidative stress would affect chromatin structure, DNA methylation, post-translational modifications of histones and DNA-binding proteins [25]. These chromatin modifications then induce various cellular alterations, such as altered gene expression, cell survival, cell death, and mutagenesis [25,26]. Hence, we examined the possible status changes in DNA methylation and histone acetylation by oxidative stress, focusing on activities of DNA methyltransferase (DNMT) and histone deacetylase (HDAC), respectively. Such studies showed that activities of both DNMT and HDAC were slightly or insignificantly lost with PL or GSP alone; however, their activities were significantly (≥50%) lost with the PL/GSP combination (Figure 4). These findings suggest that oxidative stress is indeed capable of inducing chromatin modifications, subsequently leading to a cessation of cell growth or cell death. For instance, it has been reported that such chromatin modifications would up-regulate p21<sup>WAF1</sup>, one of specific cell cycle regulators, which can induce a G<sub>1</sub> cell cycle arrest [28]. As a result, cells completely stop growing and some of them will die out. In addition, it has been also reported that the combination of GSP and resveratrol synergistically inhibited the growth of breast cancer cells through epigenetic changes with DNMT and HDAC [26]. Therefore, these reports support our finding that chromatin modifications induced by the PL/GSP combination (via oxidative stress) may also contribute to the resulting cell viability reduction.

Lastly, we examined if cell death induced by the PL/GSP combination might be linked to apoptosis because of its possible clinical application. Analysis of the two key apoptotic regulators revealed that bcl-2 (anti-apoptotic) was down-regulated while Bax (pro-apoptotic) was up-regulated with the PL/GSP combination (Figure 5), clearly indicating induction of apoptosis [27]. Some other reports have also shown that sole GSP was capable of inducing apoptosis through oxidative stress in bladder cancer cells [29] and cadmium chloride also induced apoptosis via oxidative stress in liver carcinoma cells [30]. Hence, it is well accepted that oxidative stress can induce apoptosis in various cancer cells, including bladder cancer. Now, our finding of PL/GSP-induced apoptosis is rather significant because “apoptosis” would clinically imply few side effects, opposed to necrosis with severe side effects induced by chemotherapy (and other drug treatments). Therefore, it is promising if this combination were certainly effective on actual patients with bladder cancer. Nevertheless, we plan to next perform the in vivo study using nude mice, and that would give us the valuable data and the true efficacy of this combination against tumor-bearing mice.

Conclusions
In the present study, PL and GSP individually demonstrate anticancer effect on bladder cancer T24 cells. When they were combined at the relatively low concentrations, anticancer effect was synergistically potentiated. This enhanced anticancer activity was primarily attributed to oxidative stress, chromatin modifications, and ultimate apoptosis. Therefore, the combination of PL and GSP may provide an alternative therapeutic option, particularly as an adjuvant intravesical therapy, for superficial bladder cancer. Further studies are warranted.

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