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Original Research Article

Chemosensitizing effect of maitake mushroom extract on carmustine cytotoxicity in human bladder cancer cells

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ABSTRACT

Background: Despite several therapeutic options available for bladder cancer, the outcomes are less satisfactory. To find a more effective modality, we were interested in the bioactive mushroom extract, PDF, which has been shown to sensitize certain anticancer drugs. Accordingly, we investigated if cytotoxic effects of several anticancer drugs used on bladder cancer patients could be enhanced with PDF in vitro.
Methods: Human bladder cancer T24 cells were treated with four anticancer drugs, carmustine (BCNU), 5-fluorouracil (5FU), cisplatin (CPL), and doxorubicin (DOX) alone, their combinations, or in combination with PDF, and cell viability was determined. To explore the anticancer mechanism, the status of glyoxalase I (Gly-I), an enzyme involved in the drug resistance of cancer cells, and oxidative stress that can cause severe cellular injury/damage was also assessed.
Results: BCNU and 5FU alone resulted in a >50% reduction in cell viability but CPL and DOX had no such effects. Only a combination of BCNU and PDF led to a drastic (~90%) cell viability reduction, accompanied by inactivation of Gly-I and an increase in oxidative stress. However, any combinations of other drugs and PDF had little effects on cell viability, Gly-I activity, or severity of oxidative stress.
Conclusions: This study shows that anticancer activity of BCNU is significantly potentiated with PDF in T24 cells. This is rather attributed to inactivated Gly-I and increased oxidative stress. Therefore, PDF appears to have a chemosensitizing effect capable of enhancing BCNU cytotoxicity, which may offer an alternative, improved therapeutic option for bladder cancer.

Keywords: Bladder cancer cells, Carmustine, Glyoxalase I, Maitake mushroom, Oxidative stress

INTRODUCTION

Bladder cancer is the second most common genitourinary malignancy with high morbidity and mortality rates in the United States.[1] Approximately 80,000 new cases were diagnosed and nearly 18,000 patients died in 2019.[2] Among several available therapeutic options for bladder cancer, intravesical administration of *Bacillus Calmette-Guerin* (BCG) is the most effective immunotherapy for superficial bladder cancer and carcinoma in situ (CIS).[3] However, the therapeutic benefits of BCG are sometimes outweighed by its severe side effects, limiting its use in clinical practice.[3,4]

Chemotherapy, using a variety of cytotoxic drugs including 5-flurouracil (5FU), cisplatin (CPL), doxorubicin (DOX), gemcitabine, mitomycin C, cyclophosphamide, methotrexate, vinblastine, estramustine, etc., and their various combinations are often used in bladder cancer patients. However, the efficacy of these trials is of limited duration and no significant advantage in survival has been found in patients.[5] Needless to say, palpable side effects are another drawback and a safer and more effective therapeutic modality needs to be urgently established. The inefficacy of many anticancer drugs is attributed to the multidrug resistance of cancer cells, but glyoxalase I

(Gly-I) might play a key role in overcoming such a drug resistance.[6] Gly-I is one of glutathione-dependent antioxidant enzymes to scavenge reactive oxygen species (ROS) and also detoxify cytotoxic metabolites and agents.[7] It is thus plausible that despite Gly-I being a vital antioxidant enzyme, its "inactivation" may help overcome the drug resistance, leading to the growth cessation and/or cell death in bladder cancer cells.

Carmustine (BCNU) is an anticancer drug clinically used in the treatment of brain tumor (glioma) and also known as a blocker of the redox cycling of glutathione, reducing availability of cellular GSH (reduced glutathione).[8] Hence, BCNU is believed to inactivate Gly-I, which essentially requires GSH for its activation, and such Gly-I inactivation may overcome the drug resistance of cancer cells.[7]

In addition, anticancer activity of BCNU has been reported to be potentiated with maitake mushroom extract (PDF) in prostate cancer cells, although the exact mechanism has not been fully understood.[9] PDF is the bioactive polysaccharide with β-glucan as an active component, extracted from maitake mushroom and commercially available for a variety of medical/scientific research.[10] In fact, PDF has been extensively studied and shown to have immunomodulatory and anticancer/ antitumor activities.[11,12]

Moreover, as far as the safety of PDF is concerned, it has been exempted from a phase I toxicology study and also approved by the U.S. Food and Drug Administration (FDA) for an investigational new drug (IND) application to conduct a phase II study on patients with advanced breast and prostate cancer.[13] Thus, the safety of PDF has been certainly granted.

Accordingly, we investigated whether BCNU and other anticancer drugs might have anticancer effects or any drug-PDF combinations would further potentiate their cytotoxicity on bladder cancer cells in vitro. Moreover, to have a better understanding of the anticancer mechanism of drugs or drug-PDF combinations, we also examined how Gly-I and oxidative stress would play a role in the ultimate cell viability reduction. More details and interesting findings are described and discussed herein.

METHODS

Cell culture

The human bladder cancer T24 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Maitake mushroom extract, PDF, was a generous gift from the manufacturer (Mushroom Wisdom, Inc., East Rutherford, NJ).

For experiments, cells were seeded at the initial cell density of 2×10^5 cells/ml and treated with various anticancer drugs/agents for specified times. Cell viability was then determined by MTT assay described below.

MTT assay (cell viability test)

Anticancer effect of drugs/agents tested can be assessed by determining how many cells (%) are yet alive/viable relative to control cells (100%) after drugs/agents treatment. For instance, 30% cell viability indicates that only 30% of cells are alive while the rest of 70% are dying/dead (due to anticancer activity). Cell viability was often determined 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), 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 was read on a microplate reader. Cell viability was then expressed by the percent (%) of viable cells relative to the control reading (100%).

Glyoxalase I (Gly-I) assay

T24 cell extracts were obtained by 3 cycles of freeze-thaw in liquid nitrogen and Gly-I activity was determined using the spectrophotometric method as described previously.[14] The reaction was started by the addition of cell extracts (40 μg) to the reaction mixture. The increase in absorbance at 240 nm, due to a production of S-D-lactoylglutathione, was measured with times using a spectrophotometer. Gly-I activity was then expressed by μmol/mg protein where Gly-I catalyzes the formation of 1 μmol of S-D-lactoylglutathione per minute.

Lipid peroxidation (LPO) assay

The severity of oxidative stress was assessed by the LPO assay, by measuring the amount of malondialdehyde (MDA) formed in the plasma membrane, due to oxidative stress.[15] As MDA is an end product from peroxidation of polyunsaturated fatty acids, the severity of oxidative stress can be indicated as: the more MDA formed, the greater oxidative stress. The LPO colorimetric assay kit (Abcam, Cambridge, MA) was used and the procedures were described in the vendor's protocol. The amount of MDA formed (in each sample) was then expressed by μM determined from the MDA standards.

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 ANOVA analysis. Values of $p<0.05$ were considered to indicate statistical significance.

RESULTS

Effects of various anticancer drugs on T24 cell viability

To assess the cytotoxic effects of four anticancer drugs, BCNU (50 μM), 5FU (5 μg/ml), CPL (100 μM), or DOX (5 ng/ml), T24 cells were treated individually with these drugs for 72 hours and cell viability was determined. The concentrations of these drugs used were the estimated maximum or above physiologically tolerable levels. The results showed that both BCNU and 5FU led to a >50% reduction in cell viability but CPL and DOX had no such effects (Figure 1A).

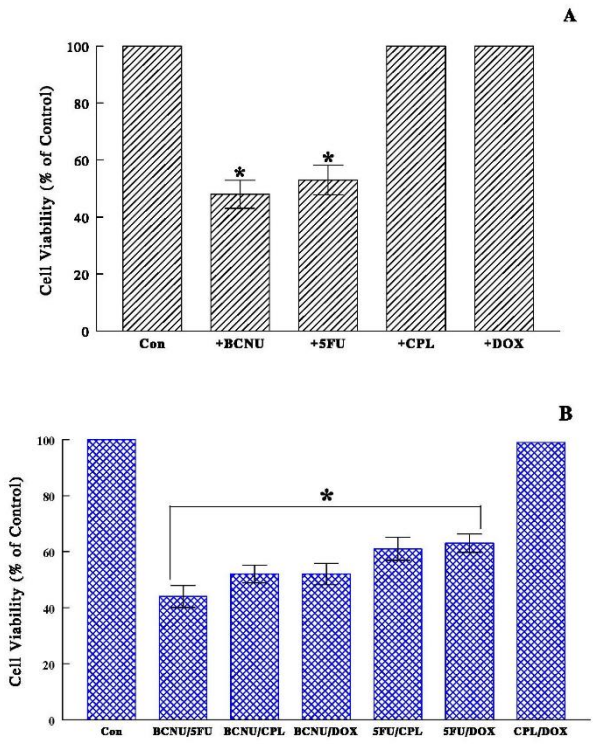

Figure 1: (A) Effects of various anticancer agents on cell viability; (B) combined effects of various anticancer agents on cell viability.

A) T24 cells were treated with BCNU (50 μM), 5-FU (5 μg/ml), CPL (100 μM), or DOX (5 ng/ml) for 72 hours. Cell viability (%) relative to controls was determined and plotted. All data are mean±SD (standard deviation) from three separate experiments (*p<0.05 compared with control). B) Cells were treated with six different combinations of two agents, such as BCNU+5FU, BCNU+CPL, BCNU+DOX, 5FU+CPL, 5FU+DOX, and CPL+DOX, for 72 hours and cell viability was determined. The data are mean±SD from three independent experiments (*p<0.05 compared with control).

The 2-drug combinations of BCNU, 5FU, CPL and DOX, such as BCNU+5FU, BCNU+CPL, BCNU+DOX, 5FU+CPL, 5FU+DOX, and CPL+DOX (6 combinations total), were tested next. However, no significant improvement (with an additive effect) in cytotoxic effect was seen in any combinations (Figure 1B). Although a significant cell viability reduction was observed in four combinations (BCNU+CPL, BCNU+DOX, 5FU+CPL, and 5FU+DOX), such a reduction was basically due to the cytotoxic effect of BCNU or 5FU. The BCNU+5FU combination led to a slightly higher/better cell viability reduction than these four combinations; however, it didn't even show an additive effect and wasn't significantly different from the viability reductions induced by BCNU or 5FU alone. Therefore, any combinations of four drugs would not significantly improve their anticancer activities.

Enhanced cytotoxic effect of BCNU in combination with PDF

Although any drug-drug combinations may not work, at least BCNU and 5FU by itself are found to have anticancer activities, and PDF has been shown to have a chemosensitizing effect on some anticancer drugs.[16] It was then reasonable to examine if a "drug-PDF" combination might improve the efficacy/cytotoxicity of BCNU or 5FU. T24 cells were treated with BCNU (50 μM) or 5FU (5 μg/ml) combined with 60 μg/ml of PDF, and cell viability was evaluated at 24 hours. Although PDF (60 μg/ml) alone had no effect, cell viability considerably declined from ~50% (with BCNU alone) to ~10% when BCNU was combined with PDF (Figure 2). In contrast, no such sensitized cytotoxic effect was seen in the 5FU/PDF combination. Thus, only BCNU appears to be synergistically potentiated with PDF, implying a selective chemosensitizing effect of PDF.

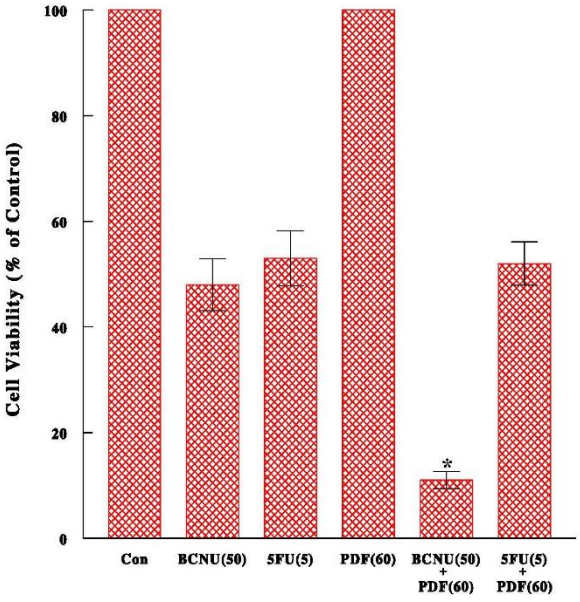

Figure 2: Combined effects of BCNU or 5FU and PDF on cell viability.

Cells were treated with BCNU (50 μM), 5FU (5 μg/ml), PDF (60 μg/ml) alone or combinations of BCNU+PDF or 5FU+PDF for 24 hours and cell viability was determined. All data are mean±SD from three separate experiments (*p<0.05; compared with BCNU).

Role of Gly-I in BCNU-induced cell viability reduction

The finding that only BCNU, not 5FU, responded to PDF to enhance its cytotoxic effect tempted us to look into Gly-I whose activity is shown to be inhibited by BCNU.[8] After cells were exposed to BCNU (50 µM), 5FU (5 µg/ml), PDF (60 µg/ml), BCNU+PDF, or 5FU+PDF for 6 hours, Gly-I activity was determined. The results showed that Gly-I activity decreased to ~65% (i.e. a ~35% decrease) with BCNU alone while 5FU and PDF had no effects (Figure 3). Yet, Gly-I activity slightly further decreased to ~60% when BCNU was combined with PDF (BCNU+PDF), although no such effect was seen with 5FU+PDF combination. Thus, these results suggest that only BCNU seems to specifically inactivate Gly-I, accounting for BCNU-induced cell viability reduction.

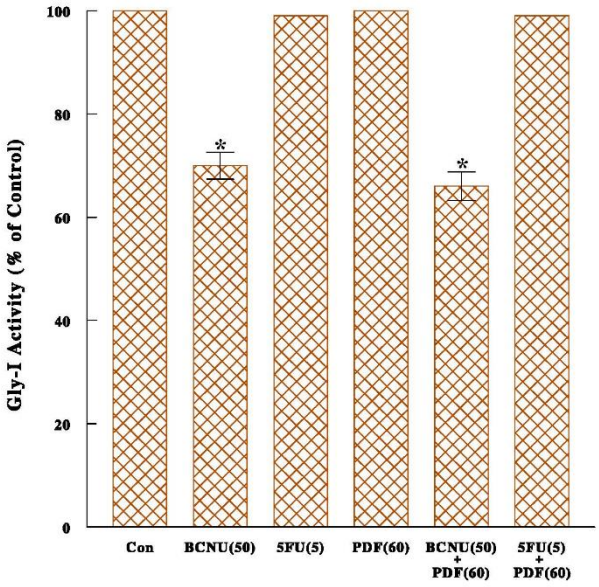

Figure 3: Effects of BCNU, 5FU or PDF on Gly-I activity.

Cells exposed to BCNU (50 µM), 5FU (5 µg/ml), PDF (60 µg/ml) alone or combinations of BCNU+PDF or 5FU+PDF for 6 hours were subjected to Gly-I assay. Gly-I activity (µmol/mg protein) was calculated and expressed by the % relative to controls (0.75 µmol/mg =100%). All data are mean±SD from three independent experiments (*p<0.03 compared with control).

To address a relationship between cell viability reduction induced by BCNU and Gly-I activity, the results of Figures 2 and 3 were plotted together as shown in Figure 4. Both cell viability and Gly-I activity significantly declined with BCNU, while 5FU led to a cell viability reduction but had no effect on Gly-I. The BCNU+PDF combination also led to a ~40% higher viability reduction (to ~10%) but no further reduction was seen with 5FU+PDF combination. Interestingly, only 5% more Gly-I inactivation was found with BCNU+PDF (compared to that with BCNU alone), despite a ~40% additional cell viability reduction. No change in Gly-I was seen between 5FU alone and 5FU+PDF combination. Thus, BCNU-induced cell viability reduction is well associated with Gly-I activity, whereas 5FU-induced reduction may follow the non-Gly-I-led mechanism.

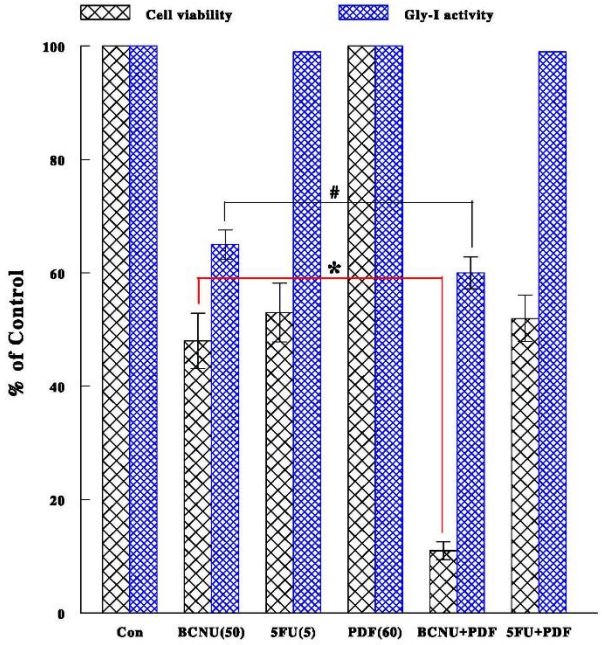

Figure 4: Relationship between BCNU-induced cell viability reduction and Gly-I activity.

The data from Figure 2 (for cell viability) and Figure 3 (for Gly-I activity) were plotted together against different experimental conditions as indicated. No statistical difference between two groups is indicated by #, while a significant statistical difference is indicated by * ($p<0.05$ between two groups).

However, there is virtually little difference (only 5%) in Gly-I inactivation between BCNU and BCNU+PDF to account for a ~40% difference in cell viability reduction (Figure 2). This suggests that some other factor might be contributing to such a profound (~90%) cell viability reduction induced by BCNU+PDF.

Increase in oxidative stress with Gly-I inactivation

Now, the question is what factor might play a significant role in BCNU+PDF-induced cell viability reduction. As Gly-I is an antioxidant enzyme, it is possible that its inactivation by BCNU could significantly increase the oxidative stress level, which may ultimately lead to a cell viability reduction.[7] To test this possibility, cells were exposed to BCNU (50 µM), 5FU (5 µg/ml), PDF (60 µg/ml), BCNU+PDF, or 5FU+PDF for 6 hours, severity of oxidative stress was assessed by LPO assay, indicating "the more MDA formed, the greater oxidative stress." As shown in Figure 5, BCNU, 5FU or PDF alone showed no statistically significant increases in the MDA levels compared to controls.

In contrast, a significant 2.3-fold increase in MDA level was observed with BCNU+PDF but no such effect was seen with 5FU+PDF. Thus, only when BCNU is combined with PDF, severe oxidative stress is exerted on

T24 cells, subsequently leading to the growth inhibition and/or cell death. In fact, this finding is consistent with a drastic (~90%) reduction in cell viability induced by BCNU+PDF (Figure 2). It is plausible that BCNU by itself may primarily target Gly-I and BCNU+PDF may also exert severe oxidative stress, thereby presumably resulting in such a profound cell viability reduction.

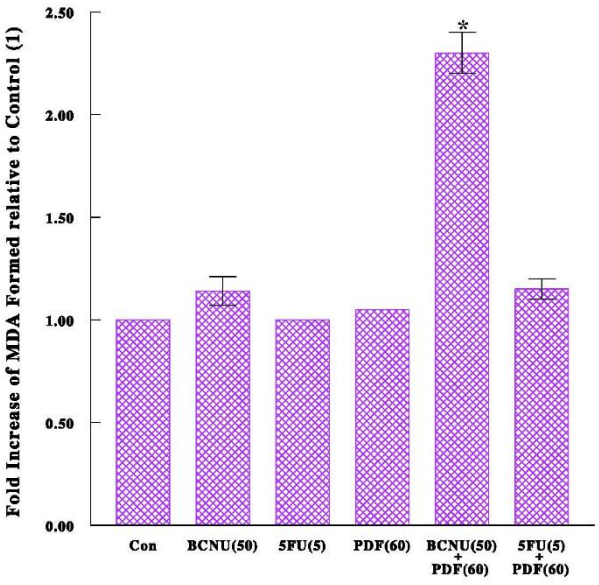

Figure 5: Exertion of oxidative stress by combination of BCNU and PDF.

Cells were exposed to BCNU (50 µM), 5FU (5 µg/ml), PDF (60 µg/ml), BCNU+PDF, or 5FU+PDF for 6 hours and severity of oxidative stress was assessed by LPO assay. Severity of oxidative stress was assessed by the amounts of MDA formed and expressed by fold-increase relative to controls (1). All data are mean±SD from three independent experiments (*p<0.01 compared with control).

DISCUSSION

Due to the ineffective therapeutic options and unsatisfactory outcomes, patients with bladder cancer 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. We then came across the bioactive component (PDF) isolated from maitake mushroom, which has been shown to have anticancer and antitumor activities.[11,16] Although chemotherapy is known to be rather ineffective with substantial side effects on bladder cancer, we yet hypothesized that some or certain anticancer drugs/agents might be potentiated with few side effects when combined with natural agents/substances with anticancer effects but few side effects.[1] Instead of combining various "drugs", which may worsen side effects without improving the efficacy, combinations of drug and natural agent could be a better choice. Hence, we investigated if any specific combinations of various drugs and PDF would improve anticancer/cytotoxic effects of those drugs.

We first examined if four clinically used anticancer drugs, BCNU, 5FU, CPL or DOX, would have any effects on cell viability in bladder cancer T24 cells. Cell viability is indicative of the % of viable cells after drug treatment. BCNU and 5FU led to a >50% reduction in cell viability (i.e. <50% viable), while CPL and DOX had no effects (i.e. 100% viable). Next, we tested the effects of 2-drug combinations of all four drugs (BCNU+5FU, BCNU+CPL, BCNU+DOX, 5FU+CPL, 5FU+DOX, and CPL+DOX) on cell viability. We found no improvements in cytotoxic effects of any combinations tested.

Despite a failure of drug-drug combinations, we further examined if BCNU and 5FU could be potentiated when combined with a non-toxic PDF. The BCNU+PDF combination led to a ~90% reduction in cell viability, while the 5FU+PDF had no such effect. It was peculiar that only BCNU, not 5FU, responded well to PDF. As BCNU is a putative inhibitor of Gly-I, its inhibition with BCNU could account for such a cell viability reduction.[8] Gly-I analysis indeed showed that BCNU led to a ~35% loss in Gly-I activity and we also found that there was a good correlation between BCNU-induced cell viability reduction and Gly-I inactivation (Figure 4). Nevertheless, no effect of 5FU was seen on Gly-I activity either.

However, it was yet puzzling when we also found that BCNU+PDF reduced Gly-I activity by ~40% while BCNU alone reduced it by ~35%. There was no significant difference in Gly-I inactivation induced with BCNU alone or BCNU+PDF. However, cell viability was reduced from ~50% with BCNU alone to ~10% with BCNU+PDF, i.e. a significant 40% difference. We then assumed that inactivation of Gly-I with BCNU could weaken an antioxidant defense against oxidative stress as Gly-I is an antioxidant enzyme.[7] We found that BCNU+PDF exerted a significantly severer oxidative stress but BCNU alone showed nearly the basal level of oxidative stress. Nevertheless, 5FU alone and 5FU+PDF had little effects on oxidative stress. Therefore, these findings suggest that BCNU+PDF-induced drastic cell viability reduction is most likely attributed to Gly-I inactivation as well as increased oxidative stress.

Since the multidrug resistance of cancer cells is a major factor for the inefficacy of various anticancer drugs/agents, inactivation of Gly-I with BCNU could be even more significant.[19] Gly-I is an antioxidant and detoxifying enzyme capable of diminishing oxidative stress and detoxifying cytotoxic drugs/substances including anticancer drugs.[6,20] Hence, a higher Gly-I activity in cancer cells may imply a possible acquisition of the drug resistant nature (with detoxification) as the cancer develops. Targeting and inactivating Gly-I could be a viable strategic approach to weaken and overcome the drug resistance and sensitize cancer cells to drugs. Thus, exploration of drugs/agents acting on Gly-I should be encouraged and actively proceeded.

Moreover, even BCNU by itself would inevitably develop side effects as its doses increase, but its combination with non-toxic agents such as PDF may

improve the drug efficacy while minimizing side effects. As a brief note to be added, a non-randomized clinical study of PDF on 165 patients with various types of advanced cancers showed that side effects of chemotherapy on all those patients were ameliorated when PDF was given with drugs simultaneously. Adverse symptoms such as nausea, hair loss and leukopenia were alleviated in 90% of patients and a reduction in pain was also reported in 83% of patients.[16] Therefore, as PDF appears to help minimize side effects of chemotherapy, it could be also considered as a natural, safe, non-toxic adjuvant agent in ongoing cancer chemotherapy.

CONCLUSION

This study demonstrates that anticancer drug, carmustine (BCNU), has a cytotoxic effect on bladder cancer T24 cells, inducing a >50% cell viability reduction. Such cytotoxicity was further potentiated with a maitake mushroom extract (PDF) resulting in a profound ~90% cell viability reduction. This was also accompanied by significant glyoxalase I (Gly-I) inactivation and increased oxidative stress. Therefore, a maitake mushroom extract may have a chemosensitizing effect to enhance carmustine cytotoxicity on bladder cancer. This finding further suggests that glyoxalase I could be a potential target for the development of novel anticancer drugs and certain drugs/agents with prooxidant activity (exerting oxidative stress) may also offer an alternative anticancer strategy against bladder cancer.

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