Chemosensitization of Carmustine with Maitake β-Glucan on Androgen-Independent Prostatic Cancer Cells: Involvement of Glyoxalase I

MICHAEL P. FINKELSTEIN, M.D., SHAHRAD AYNEHCHI, M.D., ALBERT A. SAMADI, M.D., SOPHIA DRINIS, M.D., MUHAMMAD S. CHOUDHURY, M.D., HIROSHI TAZAKI, M.D., Ph.D., and SENSUKE KONNO, Ph.D.

ABSTRACT

Objective: To improve the poor efficacy (< 10%) of chemotherapy for patients with hormone-refractory prostate cancer, we investigated a possible cytotoxic effect of carmustine/β-glucan combination on prostatic cancer PC-3 cells, focusing on a glutathione-dependent detoxifying enzyme, glyoxalase I (Gly-I).

Methods: Carmustine (BCNU) is an anticancer agent and a putative inhibitor of Gly-I, while β-glucan is a unique, nontoxic polysaccharide extracted from maitake mushrooms. The cytotoxic effects of BCNU or other anticancer agents with β-glucan on PC-3 cells were assessed by cell-viability testing and Gly-I activity was measured using the spectrophotometric method.

Results: BCNU, 5-fluorouracil (5-FU), and methotrexate (MTX) were capable of inducing approximately a 50% reduction in cell viability at 72 hours, while etoposide, cisplatin, and mitomycin C were all ineffective. Only the combination of BCNU (50 μmol) and β-glucan (60 μg/mL) exhibited an enhanced cytotoxicity with an approximate 90% cell viability reduction, but little improvement was seen with any combinations of 5-FU, MTX, or β-glucan. Gly-I assays revealed that such a profound (~90%) cell death was accompanied by an approximate 80% reduction in Gly-I activity by 6 hours.

Conclusion: This study demonstrates a sensitized cytotoxic effect of BCNU with β-glucan in PC-3 cells, which was associated with a drastic (~80%) inactivation of Gly-I. Therefore, the BCNU/β-glucan combination may help to improve current treatment efficacy by targeting Gly-I, which appears to be critically involved in prostate cancer viability.

INTRODUCTION

The majority of patients with hormone-refractory prostate cancer (PC) survive only a few years despite the availability of various treatments (Mahler and Denis, 1992). One such modality is chemotherapy using a variety of cytotoxic agents including adriamycin, cisplatin (CPL), methotrexate (MTX), 5-fluorouracil (5-FU), vinblastine, cyclophosphamide, estramustine, mitomycin C (Mit.C) etc., and their combinations. However, the efficacy of these drugs is of limited duration and no significant advantage in survival has been found for patients receiving these therapies (Theyer and Hamilton, 1994; Yagoda and Petrylak, 1993). The mul-

Department of Urology, New York Medical College, Valhalla, NY.
tidrug-resistant nature of PC is then believed to account mainly for the failure of chemotherapy with a less than 10% response rate (Yagoda and Petrylak, 1993). To improve the efficacy of chemotherapy, such drug resistance in PC must be overcome and its mechanism(s) also needs to be fully elucidated.

Among several proposed mechanisms of multidrug resistance, three mechanisms such as p-glycoprotein (Weinstein et al., 1990), topoisomerase II (Pommier, 1993), and glutathione-related enzymes (particularly glutathione S-transferase \( \pi \) ) (Tew, 1994) have mostly been studied in many human malignancies including PC. However, the primary mechanism of the drug resistance in PC yet remains elusive (Theyer et al., 1993; Theyer and Hamilton, 1994). We were then interested in glyoxalase I (Gly-I), one of glutathione-related enzymes (Thornalley, 1990), because it had been assumed to play a key role in the multidrug resistance of cancer cells (Thornalley, 1998). Gly-I is a vital detoxifying enzyme of cytotoxic metabolites and agents (Thornalley, 1998) but has been studied the least in such drug-resistance cases.

Carmustine (BCNU) is a blocker of the redox cycling of glutathione (i.e., the conversion of oxidized glutathione to its reduced form [GSH]) by inhibiting glutathione reductase (Vanhoef et al., 1997), resulting in the reduced availability of cellular GSH. BCNU is thus believed to inactivate Gly-I, which essentially requires GSH for its activation (Thornalley, 1990). Such Gly-I inactivation may then help overcome drug resistance, leading to growth cessation and/or cell death in prostatic cancer cells. Moreover, clinical trials of BCNU on patients with PC were conducted more than 20 years ago (Presant et al., 1976, 1980), and showed some promising outcomes. However, because no further studies have been reported since then, it is worthwhile to resume the investigation of BCNU to reevaluate its efficacy on prostatic cancer cells and to explore its cytotoxic mechanism.

In addition, our recent study (Fullerton et al., 2000) indicated that BCNU cytotoxicity might be potentiated with maitake \( \beta \)-glucan, although the exact mechanism remains to be elucidated. This maitake \( \beta \)-glucan is the unique polysaccharide extracted from maitake mushroom (Grifola frondosa) (Mizuno and Zhuang, 1995). Its highly purified preparation, namely Grifron™-D (GD; Maitake Products, Inc., Paramus, NJ), is commercially available for a variety of medical/scientific research. GD has been shown to have immunomodulatory and anti-tumor activities against various cancers (Adachi et al., 1987; Nanba, 1993). Recently, the U.S. Food and Drug Administration (FDA) has approved an investigational new drug (IND) application to conduct a phase II study on GD in patients with advanced breast and prostate cancer (Maitake Products, Inc., 1998). GD has also been reported to have few side-effects on normal subjects and to even alleviate severe side-effects on patients with cancer receiving chemotherapy (Nanba, 1997).

Accordingly, in this study, we explore the underlying mechanism by which the combination of BCNU and GD may potentiate/sensitize cytotoxic activity, focusing on the involvement of Gly-I in androgen-independent prostatic cancer PC-3 cells in vitro. A potential therapeutic modality using BCNU and GD for hormone-refractory prostate cancer is also discussed.

**MATERIALS AND METHODS**

**Cell culture**

Androgen-independent human prostatic cancer PC-3 cells (Kaighn et al., 1979), derived from the bone metastatic site of a patient with PC, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were maintained in RPMI-1640 medium with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 \( \mu \)g/mL). For experiments, cells were seeded in 6-well plates or T-75 flasks at the initial cell density of \( 1 \times 10^5 \) cells per milliliter and were cultured with specified concentrations of various anticancer drugs/agents including BCNU and \( \beta \)-glucan (GD). BCNU was reconstituted in ethanol to give a stock concentration of 250 mmol/L, which was used with appropriate dilutions if necessary. Nevertheless, ethanol concentration has never exceeded 0.08%, which had no cellular effects. A stock GD solution was prepared at 20 mg/mL in
water and sterilized through a 0.2-μm sterile filter (Nalgene, Rochester, NY). At the given concentrations (30 or 60 μg/mL) of GD, neither pH change in culture media nor precipitation of GD in cell cultures was observed. Cell viability was determined using the AlamarBlue cell viability test (Biosource International, Camarillo, CA). Anticancer agents were purchased from Sigma Chemical Co. (St. Louis, MO) or Calbiochem (La Jolla, CA), and the GD was a kind gift from Maitake Products, Inc. (Paramus, NJ).

Collection of clinical prostate specimens

Prostate tissue specimens were freshly obtained from 15 patients with prostate cancer (PC) undergoing radical prostatectomy and from 5 patients with benign prostatic hyperplasia (BPH) at transurethral resection of the prostate. A selected portion of each specimen was excised and sent for histologic examination, while its adjacent section was stored at −80°C. Based on the pathology report, a total of 28 specimens were chosen for this study: 15 PC and 13 noncancerous cell specimens including 8 uninvolved portions (negative for cancer) from PC and 5 BPH specimens.

Gly-I assay

PC-3 cell extracts were obtained by cell lysis in liquid nitrogen, while tissue homogenates were prepared from a small portion (~50 mg) of prostate tissue that had been homogenized using a tissue grinder. Gly-I activity was measured using the spectrophotometric method as described previously (Ranganathan and Tew, 1993). After preparation of the reaction mixture (200 mmol/L imidazole HCl, pH 7.0, 16 mmol/L MgSO₄, 7.9 mmol/L methylglyoxal, 1 mmol/L GSH), the reaction was started by the addition of cell extracts or tissue homogenates (40 μg). The increase in absorbance at 240 nm, because of production of S-δ-lactoylglutathione (E₂₄₀ = 3.37 mmol/L⁻¹ cm⁻¹), was measured with times using a spectrophotometer. Gly-I activity was then expressed by micromole per milligram of protein or units per milligram of protein where 1 unit is defined to catalyze the formation of 1 μmol of S-δ-lactoylglutathione per minute.

Statistical analysis

Statistical differences in the experimental data were assessed using the unpaired Student's t test. A value of p < 0.05 was considered significant.

RESULTS

Effects of BCNU and various anticancer agents on prostate cancer viability

We first assessed a potential cytotoxic effect of BCNU on prostatic cancer PC-3 cells that had been cultured with the varying concentrations

![Figure 1A](image1.png)

**FIG. 1.** A: Dose-response effects of BCNU on cell viability. PC-3 cells were cultured with 0, 50, 100, 200, and 300 μmol/L of BCNU and cell viability was determined at 24 hours. The data are mean ± standard deviation (SD) from three independent experiments. B: Effects of various anticancer drugs on cell viability. Cells were treated with BCNU (50 μmol/L), 5-FU (5 μg/mL), MTX (100 μmol/L), VP-16 (100 nmol), CPL (100 μmol/L), or Mit.C (300 nmol) for 72 hours. Cell viability (%) relative to controls was determined and plotted. All data are mean ± SD from three separate experiments. BCNU, carmustine; PC-3, human prostatic cancer cells; 5-FU, 5-fluorouracil; MTX, methotrexate, VP-16, etoposide; CPL, cisplatin; Mit.C, mitomycin C.
(0–300 μmol/L) of BCNU for 24 hours. This dose-dependent study revealed that cell viability was reduced by approximately 50% with 50 μmol/L BCNU, declined to approximately 10% at 100 μmol/L, and resulted in nearly complete cell death at BCNU 200 μmol/L or more (Fig. 1A). Thus, BCNU appears to have a potent cytotoxic effect (with the IC50 = 50 μmol/L) on PC-3 cells.

For comparison with BCNU, similar study was performed with several anticancer agents currently used in prostate cancer treatment (Yagoda and Petrylak, 1993), such as 5-FU (5 μg/mL), MTX (100 μmol/L), etoposide (VP-16, 100 nmol), CPL (100 μmol/L), and Mit.C (300 nmol). The concentrations of these agents used were the estimated maximum or above physiologically tolerable levels. After cells were treated with these agents (including 50 μmol/L BCNU as a reference) for 72 hours, cell viability test was performed. Both 5-FU and MTX were found to induce approximately a 50% reduction in cell viability similar to that attained with BCNU, whereas VP-16, CPL, and Mit.C had no such effect (Fig. 1B).

Because these findings suggested that the combinations of BCNU, 5-FU, and MTX might further potentiate an individual cytotoxicity, such possibility was tested next. However, any combinations of BCNU (50 μmol/L), 5-FU (5 μg/mL), and MTX (100 μmol/L) failed to enhance their cytotoxicity, and cell viability yet remained at approximately 50% (Table 1). Thus, these three agents are unable to exhibit a synergistic or additive effect with their combinations.

### Sensitized cytotoxic effect of BCNU combined with β-glucan (GD)

To find an alternative way to improve the efficacy of three agents (BCNU, 5-FU and MTX), we examined the combined effects of β-glucan (GD) and these agents. GD is a bioactive polysaccharide of maitake mushroom (Mizuno and Zhuang, 1995), which has been postulated to have a sensitizing effect on some anticancer drugs (Nanba, 1997). PC-3 cells were cultured with three agents in combination with 30 or 60 μg/mL GD, and cell viability was evaluated at 24 hours. No cytotoxic effect was seen with GD (30 or 60 μg/mL) alone; however, when BCNU (50 μmol/L) was combined with them, cell viability declined from approximately 50% (BCNU alone) to approximately 33% (with 30 μg/mL GD) or approximately 10% (with 60 μg/mL GD) (Fig. 2). In contrast, no such sensitization of cytotoxicity was observed in 5-FU or MTX with GD (data not shown). Thus, only a cytotoxicity of BCNU was significantly potentiated or enhanced with GD, implying a selective chemosensitizing effect of GD.

### Significance of Gly-I in clinical prostate specimens

To explore the underlying mechanism of BCNU-induced or BCNU/GD-potentiated cell death, we assumed that Gly-I, a detoxifying enzyme, might be primarily affected by BCNU, a putative inhibitor of Gly-I (Vanhoefer et al., 1997). First, to elicit a significance of Gly-I in prostate cancer, Gly-I activity was assessed on clinical prostate specimens including PC, BPH,

### Table 1. Effects of Combinations of BCNU, 5-FU, and MTX on PC-3 Cell Viability

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cell viability (% of control)(^a) at 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>+ BCNU (50 μmol/L)</td>
<td>48 ± 4.9</td>
</tr>
<tr>
<td>+ 5-FU (5 μg/mL)</td>
<td>53 ± 5.2</td>
</tr>
<tr>
<td>+ MTX (100 μmol/L)</td>
<td>55 ± 3.6</td>
</tr>
<tr>
<td>+ BCNU/5-FU</td>
<td>44 ± 3.9</td>
</tr>
<tr>
<td>+ BCNU/MTX</td>
<td>46 ± 4.1</td>
</tr>
<tr>
<td>+ BCNU/5-FU/MTX</td>
<td>44 ± 3.3</td>
</tr>
</tbody>
</table>

\(^a\)The data are mean ± SD (standard deviation) from three separate experiments. BCNU, carmustine; 5-FU, 5-fluorouracil; MTX, methotrexate; PC-3, human prostatic cancer cells.
and normal (NML) prostate tissues. Gly-I assays on 15 PC and 13 noncancerous prostate (NCP, BPH, and NML) specimens showed that Gly-I activities (0.9–2.9 $\mu$mol/mg protein; mean 2.4 ± 0.28) in all PC specimens was significantly (eightfold) higher than those (0.1–0.5 $\mu$mol/mg protein; mean 0.3 ± 0.06) in noncancerous cell specimens (Fig. 3). These results suggest that an increase in Gly-I activity could be associated with the development/progression of clinical prostate cancer.

**DISCUSSION**

The failure of chemotherapy in the treatment of hormone-refractory or advanced PC is primarily because of the multidrug-resistant nature of prostatic cancer cells (Theyer and Hamilton, 1994; Yagoda and Petrylak, 1993). Despite extensive research, the exact mechanism(s) of drug resistance in PC remains elusive (Theyer et al., 1993; Theyer and Hamilton, 1994). Gly-I has been proposed to play a role in BCNU-induced cell death.

![FIG. 2. Combined effects of BCNU and GD on cell viability. Cells were cultured with 50 $\mu$mol/L BCNU alone or its combinations with 30 or 60 $\mu$g/mL GD for 24 hours and cell viability (%) was assessed. The data are mean ± standard deviation, and differences in the values between BCNU alone and its combinations with GD are statistically significant (*p < 0.05; **p < 0.01). BCNU, carmustine; GD, Grifron™-D (Maitake Products, Inc., Paramus, NJ).](image1)

![FIG. 3. Gly-I activity in prostate specimens. Gly-I activities in 15 PC and 13 NCP (BPH and NML) specimens were determined as described in Materials and Methods and were plotted against PC and noncancerous cells, respectively. *Mean ± standard deviation. Gly-I, glyoxalase; PC, prostate cancer; BPH, benign prostatic hyperplasia; NML, normal prostate tissue.)](image2)
pivotal role in detoxification of cytotoxic metabolites/agents (Thornalley, 1998) but has not been thoroughly characterized. We therefore investigated a possible involvement/role of Gly-I in cell viability of prostate cancer using carmustine (BCNU), a putative inhibitor of Gly-I (Vanhoefer et al., 1997).

We first examined the cytotoxic effect of BCNU on prostatic cancer PC-3 cells. BCNU exhibited a potent cytotoxicity, leading to almost complete cell death at the concentrations 200 μmol/L or more with the IC_{50} of approximately 50 μmol/L. For comparison, various anticancer drugs currently in use were also examined: 5-FU and MTX were capable of inducing a maximum of approximately 50% reduction in cell viability while the rest of drugs was ineffective. However, any drug combinations tested have failed to demonstrate an improved cytotoxic effect, suggesting that a different approach is required for enhancing their cytotoxicity. In addition, the multiple drug combinations are known to often have severe side-effects as well. To improve the efficacy of drugs while minimizing their side-effects, we examined the combinations of these drugs with an agent having the least side effects such as the mushroom β-glucan (GD). These studies showed that the combination of BCNU (50 μmol/L) and GD (60 μg/mL) was capable of drastically lowering cell viability to approximately 10% (an ~90% viability reduction). This enhanced BCNU cytotoxicity with GD likely reflects a sensitizing effect of GD, because the given concentrations of GD alone had no cytotoxic effect (Fig. 2). It is thus possible that further exploration of combinations of “cytotoxic drugs” with “nontoxic agents” may offer a more effective treatment modality for hormone-refractory PC.

To have an insight into the mechanism of BCNU (GD)-induced cell death, a possible involvement of Gly-I was also explored. BCNU inhibits the redox cycling of glutathione (Vanhoefer et al., 1997), limiting the availability of

FIG. 4. Effects of BCNU and GD on Gly-I activity. Cells were exposed to BCNU (50 μmol/L) or in combination with GD (30 or 60 μg/mL) for 6 hours. Cell extracts were prepared and assayed for Gly-I activity. All data are mean ± standard deviation, and differences in the values between BCNU alone and its combinations with GD are statistically significant (*p < 0.05; **p < 0.03). BCNU, carmustine; Gly-I, glyoxalase; GC, Grifron™-D (Maitake Products, Inc., Paramus, NJ).

FIG. 5. Relations between cell viability and Gly-I activity. Dose-dependent effects of BCNU (0–200 μmol/L) on cell viability (at 24 hours) and Gly-I activity (at 6 hours) were determined as the percentage relative to controls and plotted together for comparison. BCNU, carmustine; Gly-I, glyoxalase.
GSH, thereby presumably leading to the inactivation of Gly-I or other GSH-dependent enzymes as well. Our study confirmed that Gly-I activity was significantly inhibited by BCNU alone and BCNU/GD combinations within 6 hours (Fig. 4), resulting in a dramatic reduction in cell viability by 24 hours (Fig. 2). It should be noted that both 5-FU and MTX with approximately 50% cell killing (Fig. 1B) had little effect on Gly-I activity (data not shown), indicating that the cytotoxic mechanisms of 5-FU and MTX are irrelevant to Gly-I. Thus, BCNU (GD)-induced cell death appears to be tightly linked to the inactivation of Gly-I, suggesting that Gly-I may play a substantial role in cell viability of prostate cancer. Such a critical involvement of Gly-I in PC is supported further by compelling evidence that Gly-I activity is consistently higher in PC than in NCP specimens. Because a major function of Gly-I is to detoxify cytotoxic substances (Thornalley, 1998), a higher Gly-I activity in PC specimens may imply a possible acquisition of the drug resistant (detoxification) nature as the cancer develops. Thus, alterations in Gly-I activity in the prostate could be considered a useful biochemical indicator for assessing the PC development and/or progression (using the prostate biopsy specimens).

It should be also noted that clinical trials of 5-FU and MTX on patients with metastatic PC showed the disappointing response rate of less than 7% (Wozniak et al., 1993), although our in vitro study showed an approximate 50% viability reduction with these agents. This discrepancy is probably due to the exceeding physiological concentrations of 5-FU and MTX used in vitro and/or to the inherent difference between in vivo and in vitro conditions. To further validate the cytotoxic effects of BCNU (GD) or define the actual role of Gly-I in vivo, animal study is currently in progress.

CONCLUSION

BCNU has a cytotoxic effect on androgen-independent prostatic cancer cells. Its moderate cytotoxicity at 50 μmol/L (IC50) is further sensitized or potentiated in combination with β-glucan (GD), accompanied by the extensive Gly-I inactivation. This suggests that Gly-I could be considered a potential target for the development of novel anticancer drugs such as BCNU. Therefore, further investigations on other potential drug/agent combinations acting on Gly-I may provide an important clue to an effective, alternative modality for the treatment of hormone-refractory prostate cancer.

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Address reprint requests to:
Sensuke Konno, Ph.D.
Department of Urology
New York Medical College
Munger Pavilion 4th Floor
Valhalla, NY 10595

E-mail: sensuke_konno@nymc.edu