Selective Activation of Apoptosis Program by *S-p*-bromobenzylglutathione Cyclopentyl Diester in Glyoxalase I-overexpressing Human Lung Cancer Cells[1]

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ABSTRACT

***Purpose*: Glyoxalase I (GLO1) is an enzyme that plays a role in the detoxification of methylglyoxal, a side-product of glycolysis. We previously reported that GLO1 was a resistant factor to antitumor agent-induced apoptosis, and that *S-p*-bromobenzylglutathione cyclopentyl diester (BBGC), an effective inhibitor of GLO1, selectively sensitized to etoposide the drug-resistant human leukemia cells that overexpressed GLO1. In this study, we quantitatively measured GLO1 enzyme activity in various human solid tumor cells, and the antiproliferative effect of the GLO1 inhibitor was examined.**

***Experimental Design:* BBGC-induced apoptosis was assessed by flow cytometry. To evaluate antitumor activity of BBGC *in vivo*, we developed human cancer xenografts in nude mice.**

***Results:* We found that GLO1 enzyme activity was higher in all of the 38 human cancer cell lines that we examined than in the normal tissue samples. Moreover, GLO1 activity was frequently elevated in human lung carcinoma cells. Positive correlation between cellular GLO1 activity and BBGC sensitivity was observed in the lung cancer cell lines. Human lung cancer NCI-H522 and DMS114 cells, expressing higher GLO1 activity, underwent apoptosis when treated with BBGC, whereas A549 cells, expressing lower activity, did not. BBGC induced the activation of the stress-activated protein kinases c-Jun NH_2-terminal kinase 1 (JNK1) and p38 mitogen-activated protein kinase (MAPK), which led to caspase activation in GLO1-overexpressing tumor cells. BBGC significantly inhibited the growth of xenografted DMS114 and human prostate cancer DU-145.**

***Conclusions:* Our present results indicate that GLO1 is a tumor-specific target enzyme especially in human lung carcinoma cells and that the GLO1 inhibitor is a potent chemotherapeutic agent to repress GLO1-overexpressing human tumors.**

INTRODUCTION

GLO1[3] is an essential component in pathways leading to the detoxification of MG, one of the side products of glycolysis. This enzyme system is ubiquitously distributed in all mammalian cells and is involved in tissue maturation and cell death (1). Abnormal expression or activity of GLO1 has been demonstrated in some human tumors including colon and prostate (2, 3). Moreover, we have reported that GLO1 was frequently overexpressed in antitumor agent-resistant human leukemia cells, and that overexpression of GLO1 confers resistance to such antitumor agents as etoposide and Adriamycin (4). These observations indicate that the increase of GLO1 expression is closely associated with carcinogenesis (2, 3) and drug resistance (4).

BBGC, as well as *S*-(*N*-aryl-*N*-hydroxycarbamoyl) glutathione diethyl ester, is a potent cell-permeable inhibitor of GLO1 (5, 6). Our previous report demonstrated that BBGC sensitized drug-resistant leukemia cells to etoposide (4). Also, the inhibitor itself was reported to have antiproliferative activity in human leukemia cells. These results indicated that GLO1 inhibitors have potential as therapeutic agents against human leukemia cells. In solid tumors, generally refractory to antitumor agents, however, the effect of GLO1 inhibitors is still unclear.

In this study, we found that GLO1 activity was frequently elevated in lung cancer cell lines and that BBGC, an effective inhibitor of GLO1, selectively activated the apoptosis program in human lung cancer cells that overexpressed GLO1. The results indicated that BBGC shows selective antitumor activity *in vivo* with little toxicity to the mice.

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[3] The abbreviations used are: GLO1, glyoxalase I; BBGC, *S-p*-bromobenzylglutathione cyclopentyl diester; JNK1, c-Jun NH_2-terminal kinase 1; Z-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide); MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Z-Asp-CH_2-DCB, benzyloxycarbonyl-Asp-CH_2OC(O)-2,6-dichlorobenzene; Z-DEVD-fmk, *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp fluoromethylketone; MAPK, mitogen-activated protein kinase; GST-c-jun, glutathione *S*-transferase-c-jun; MG, methylglyoxal.

MATERIALS AND METHODS

Materials. Cytoplasmic protein from human normal tissue was purchased from BioChain Institute, Inc. (San Leandro, CA). BBGC was synthesized, as described previously (5, 7, 8). Z-Asp-CH_2-DCB and Z-DEVD-MCA were purchased from Peptide Institute, Inc (Osaka, Japan). Z-DEVD-fmk was purchased from Takara Co. (Tokyo, Japan).

Cell Lines and Cell Culture. The following human cancer cell lines were obtained from the National Cancer Institute (Frederick, MD) and elsewhere: lung cancer, NCI-H23, NCI-H226, NCI-H522, NCI-H460, A549, DMS273, and DMS114; colon cancer, HCC-2998, KM-12, HT-29, HCT-15, and HCT-116; stomach cancer, St-4, MKN-1, MKN-7, MKN-28, MKN-45, and MKN-74, breast cancer, MCF-7, HBC-4, BSY-1, HBC-5, and HTB-26; ovarian cancer, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SK-OV-3; brain cancer, U251, SF268, SF295, SF539, SNB-75, and SNB-78; renal cancer, RXF-631 L and ACHN; and prostate cancer, DU-145 and PC-3. All of the cells were cultured in RPMI 1640 (Nissui Co., Ltd., Tokyo, Japan) supplemented with 5% heat-inactivated fetal bovine serum and 100 μg/ml kanamycin in a humidified atmosphere of 5% CO_2 and 95% air.

GLO1 Assay. Cytosolic fractions were prepared as described previously (9). Briefly, the cells were lysed in PBS containing 1 mM phenylmethylsulfonyl fluoride by freezing and thawing and sonication and then were centrifuged at 12,000 × *g* for 20 min. The supernatant was used as the cytosolic fraction. The GLO1 assay was performed in 7.9 mM MG, 1 mM glutathione, 14.6 mM magnesium sulfate, and 182 mM imidazole-HCl (pH 7.0). Increase in absorption at 240 nm, attributable to the formation of *S*-D-lactoylglutathione, was measured with each cytosolic fraction.

Flow Cytometry. After drug treatment, harvested cells were fixed in 70% ethanol. After treatment with 2 mg/ml RNase, the cells were stained in 50 μg/ml propidium iodide solution and then analyzed using a Becton Dickinson FACScan flow cytometer (Braintree, MA).

Measurements of Cell Growth Inhibition. The sensitivity of tumor cell lines to drugs was evaluated by cell growth inhibition after 48-h incubation with BBGC. The number of viable cells was estimated by the MTS method (10) using CellTiter 96 AQ_{ueous} One Solution cell proliferation assay (Promega, Tokyo, Japan). The IC_{50} values were determined graphically from growth inhibition.

Measurement of Caspase Activity. Drug-treated cell lysates were incubated with 20 mM of Z-DEVD-MCA at 37°C for 120 min, and the release of amino-4-methylcoumarin was monitored by a spectrofluorometer (F-2500; Hitachi, Tokyo, Japan), using an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

Western Blot Analysis. Western blot analysis was performed using an anti-cleaved caspase-9 polyclonal antibody (Cell Signaling Technology, Beverly, MA), p38 (New England Biolabs, Beverly, MA), phospho-p38 (New England Biolabs) and JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA) according to the instructions of the manufacturer. Briefly, cell lysates were electrophoresed by SDS-PAGE and then transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with primary antibody for 2 h at 25°C. Detection was accomplished using an antimouse immunoglobulin that was peroxidase-conjugated and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Tokyo, Japan).

Kinase Assay. To prepare extracts, cells were lysed in a buffer solution containing 20 mM Tris-HCl (pH7.5), 150 mM $MgCl_2$, 12 mM β-glycerophosphate, 5 mM EGTA, 1% Triton X-100, 1% deoxycholate, 1 mM DTT, 1 mM sodium orthovanadate, 10 mM NaF, and 1 mM phenylmethylsulfonyl fluoride. Cell extracts were clarified by centrifugation at 15,000 × *g* for 10 min. p38 MAPK (p38) activity was assayed by Western blot analysis using phospho-specific p38 antibody (New England Biolabs) according to the manufacturer's protocol. For the JNK kinase activity, we used an immune complex kinase assay, as described previously (11). Briefly, the cellular extract was incubated with protein A-Sepharose (Zymed, San Francisco, CA) and anti-JNK1 for 2 h at 4°C. Immunoprecipitates were washed and assayed for kinase activity at 37°C for 20 min using GST-c-jun (1–169; Upstate Biotechnology, Lake Placid, NY) as a specific substrate. Protein in the reaction was resolved by SDS-PAGE and subjected to autoradiography.

Antitumor Activity against Xenografts. Lung cancer (DMS114) and prostate cancer (DU-145) were used to evaluate antitumor activity of BBGC *in vivo*. They were grown as s.c. tumors in nude mice (Charles River Laboratories, Yokohama, Japan). The mice were inoculated s.c. with a 3 × 3 × 3-mm tumor fragment. Therapeutic experiments (five mice per group) were started (day 0) when the tumor reached 50–150 mm^3. To prepare the BBGC sample solution, BBGC was first dissolved in cremophor EL/DMSO (1:1), and then diluted with a saline solution just before injection. BBGC was administrated i.p. at doses of 100 mg/kg/d from days 0 to 8. Control animals received the same volume of a saline solution. The length (*L*) and width (*W*) of the tumor mass were measured, and the tumor volume (*TV*) was calculated as: $TV = (L \times W^2)/2$. Statistical evaluations were performed using the Student's *t* test.

RESULTS

GLO1 Activity in Human Cancer Cell Lines. Previously, we demonstrated that GLO1 activity was significantly elevated in antitumor agent-resistant leukemia cells (4). Moreover, it was reported that some tumor tissue expressed higher GLO1 activity than normal tissue (2, 3). However, it is still unclear whether GLO1 activity is expressed in various human solid tumor cells. To determine this, we first examined GLO1 activity in 38 human solid tumor cell lines used in an anticancer drug screening program at our Center. As shown in Fig. 1, GLO1 activity was detected in various tumor cells at higher levels than in the human normal tissue samples. What was important was that lung carcinoma cells frequently showed higher GLO1 activity than did other cancer cells. Among the lung cancer cell lines, NCI-H522 cells showed the highest GLO1 activity (Table 1). As for other cancer cell lines, GLO1 activity was also relatively high in two prostate cancer cells and three of five colon cancer cells as compared with other cancer cell lines. These results indicated that GLO1 was generally

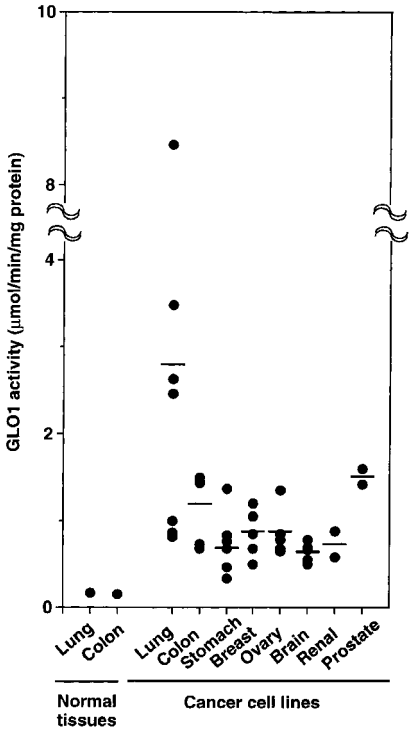

Fig. 1 GLO1 enzyme activity in 38 human solid tumor cell lines and human normal lung tissue samples. Cytosolic fractions were isolated, and GLO1 enzyme assays were performed as described in "Materials and Methods." *Bars*, means of each cell line.

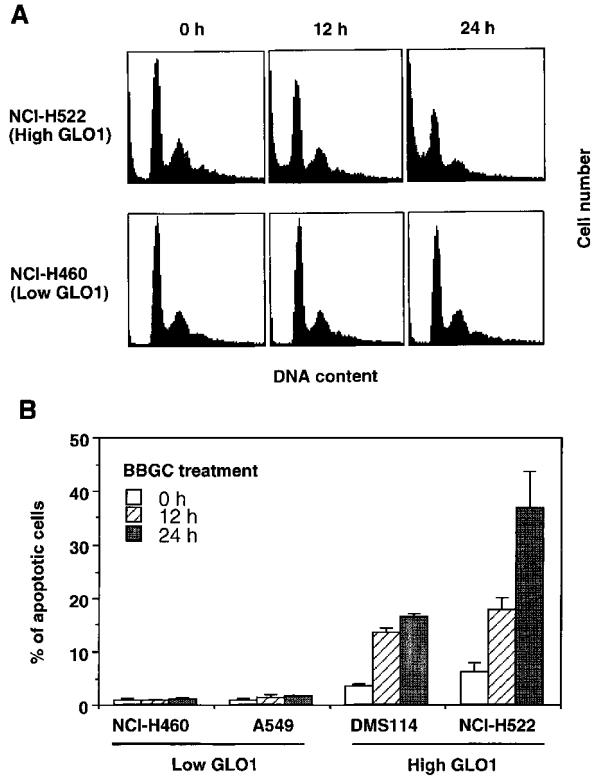

Fig. 2 Induction of apoptosis by BBGC preferentially in cells expressing a higher activity. In *A*, cells were treated with 15 μM BBGC for the indicated times. Cells were harvested and then stained with propidium iodide, and analyzed in a FACScan flow cytometer. In *B*, the distribution of sub-G_1 apoptotic cells was determined by FACScan analysis. The data represent three independent examinations.

Table 1 GLO1 activity and the sensitivity to BBGC in lung cancer cell lines

Cell lines	GLO1 activity[a] (μmol/min/mg protein)	IC_{50} to BBGC[b] (μM)
NCI-H460	0.81 ± 0.02	28.8 ± 0.7
NCI-H226	0.86 ± 0.04	25.1 ± 0.3
A549	0.99 ± 0.04	29.7 ± 1.2
NCI-H23	2.47 ± 0.03	18.9 ± 0.6
DMS273	2.63 ± 0.03	7.8 ± 0.5
DMS114	3.48 ± 0.03	7.5 ± 0.7
NCI-H522	8.47 ± 0.10	4.4 ± 0.2

[a] Cytosolic fractions were isolated, and enzyme assays were performed as described in "Materials and Methods." Values are mean ± SD determined in triplicate.

[b] Cells were treated with BBGC for 48 h. IC_{50} values of BBGC were estimated by using MTS assay. Values are mean ± SD from three independent experiments.

elevated in human carcinoma cells over normal cells and that lung cancer cells frequently expressed high GLO1 activity.

Preferential Induction of Apoptosis by BBGC in Cells Expressing Higher GLO1 Activity. Our previous report indicated that BBGC, a cell-permeable inhibitor of GLO1, showed selective antiproliferative effects on human leukemia cells that overexpress GLO1 (4). To test whether BBGC is also effective in suppressing growth of human solid tumor cells, we examined the antiproliferative effect of BBGC on human lung cancer cell lines. As shown in Table 1, BBGC preferentially suppressed growth of cells that expressed higher GLO1 activity. A significant correlation ($r^2 = 0.76$) was observed between cellular GLO1 enzyme activity and logarithms of IC_{50} values of BBGC. To examine the effect of BBGC on cell cycle progression and apoptosis, we also performed a flow-cytometric analysis. We found that, on treatment with BBGC, apoptosis was induced in NCI-H522 and DMS114 cells, which expressed higher GLO1 activity (Fig. 2*A*), but BBGC could not induce apoptosis in NCI-H460 and A549 cells, which express lower activity (Fig. 2*B*). These results indicated that BBGC selectively induced apoptosis in human lung carcinoma cells with higher GLO1 activity, and that the antiproliferative effect was closely related to the expression level of GLO1.

Activation of Caspases and Stress-activated Protein Kinases during BBGC-induced Apoptosis. The apoptosis program is generally executed by activated cysteine proteases, caspases; however, some recent reports have shown the cell death program to be independent of caspases (12, 13). Next, we determined whether activation of caspase would be involved in BBGC-induced apoptosis. The activity of caspase was measured using the fluorogenic substrate, Z-DEVD-MCA. In NCI-H522 and DMS114 cells, which showed higher GLO1 activity, caspase activity dramatically increased after BBGC treatment

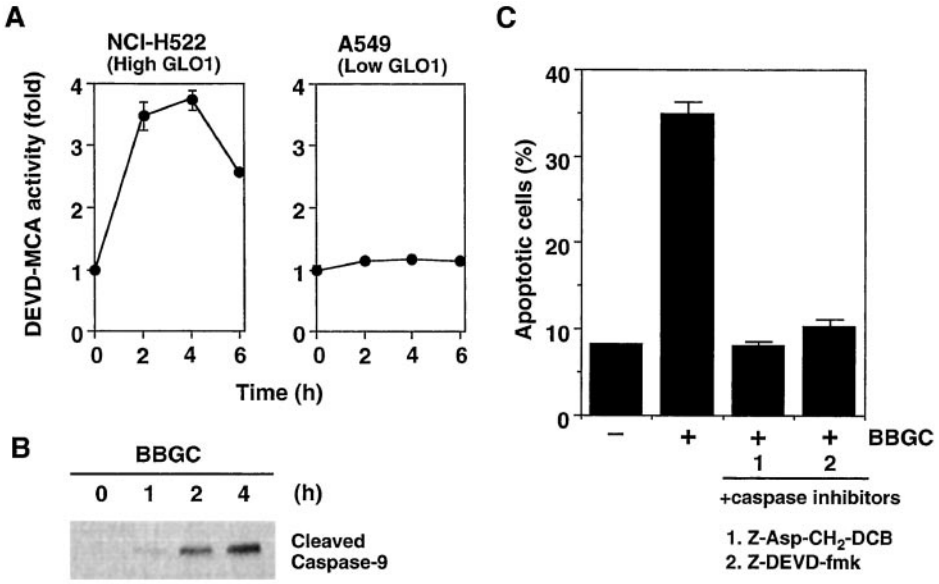

Fig. 3 Involvement of caspase activation in BBGC-induced apoptosis. In *A*, cells were treated with 15 μM BBGC for the indicated periods, and caspase-3-like protease activity was measured with the specific fluorogenic substrate Z-DEVD-MCA. In *B*, NCI-H522 cells were treated with 15 μM BBGC for the indicated periods, and the cleavage of procaspase-9 was examined by Western blot analysis using anti-cleaved caspase-9 antibody. In *C*, NCI-H522 cells were treated with 15 μM BBGC for 12 h, in the presence of caspase inhibitors Z-Asp-CH_2-DCB and Z-DEVD-fmk. The percentages of apoptotic cells were determined as subdiploid cells by flow cytometric analysis after staining with propidium iodide. The means ± SD of triplicate cultures are shown.

(Fig. 3*A*; and data not shown). Also, the processing of caspase-9, one of upstream caspases, was detected in NCI-H522 cells (Fig. 3*B*). The induction of apoptosis caused by BBGC was completely prevented by caspase-specific inhibitors, Z-Asp-CH_2-DCB and Z-DEVD-fmk, which indicated that caspase activation are required for BBGC-induced apoptosis (Fig. 3*C*). However, the activation of caspases was not observed in A549 cells, which showed lower GLO1 activity. These results indicate that caspases were selectively activated after BBGC treatment in cells that overexpress GLO1.

In stress-induced apoptosis, such stress-activated protein kinases as JNK and p38 MAPK are shown to be involved in the apoptosis signaling leading to caspase activation (14). To evaluate the role of the stress-activated protein kinases in BBGC-induced apoptosis, we examined whether BBGC is also capable of activating the p38 and JNK pathways. Fig. 4 shows that levels of phosphorylated p38 were increased after treatment with BBGC for 1 h. JNK activity increased from 1 h after treatment with BBGC. However, these activations were not observed in A549 cells, which were resistant to BBGC-induced apoptosis. Further, we found that the caspase inhibitor Z-Asp-CH_2-DCB did not inhibit activation by BBGC. These results indicated that the difference in apoptosis inducibility between the cell lines could be caused by the differential activation of the upstream kinases.

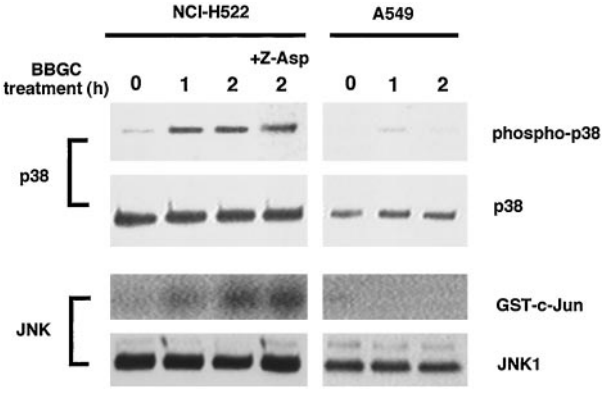

Fig. 4 p38 and JNK activation by BBGC. NCI-H522 and A549 cells were treated with 15 μM BBGC in the absence or presence of 100 μg/ml Z-Asp-CH_2-DCB for the indicated periods of time. p38 activity was measured by immunoblot analysis, with anti-phospho-specific p38 or anti-p38 antibodies. Cellular extracts were prepared and immunoprecipitated with anti-JNK antibody. JNK kinase assay was performed by using bacterially produced GST-c-Jun fusion protein as a substrate.

***In Vivo* Antitumor Activity of BBGC against Human Cancer Xenografts.** To evaluate antitumor activity of BBGC *in vivo*, we inoculated nude mice with cells with high GLO1 activity from human cancer xenografts. Because we were not successful in establishing NCI-H522 tumors, which showed highest GLO1 activity, in nude mice, we injected DMS-114 cells with the second-highest GLO1 activity, and prostate cancer DU-145 cells with the highest GLO1 activity among the cells except for lung cancer cells. Mice were given BBGC at 100 mg/kg/d daily from days 0 to 8. As shown in Fig. 5, *top panel*), treatment with BBGC significantly inhibited the growth of DMS-114 and DU-145 xenografts compared with control treatment from day 6 to 20 ($P < 0.05$). GLO1 activity was higher in human lung cancer DMS114 cells (GLO1 activity was 3.5 μmol/min/mg protein), compared with prostate cancer DU145 cells (1.6 μmol/min/mg protein). In accordance with this data, treatment with BBGC *in vivo* more strongly inhibited the growth of DMS-114 xenografts, compared with DU145. No toxic death was observed in BBGC treatment up to 200 mg/kg/d. We observed no decrease in the body weight of BBGC-treated mice throughout these experiments (Fig. 5, *bottom panels*). Taken together, these results indicate that BBGC had potent antitumor activity *in vivo* with minimal toxicity to the mice.

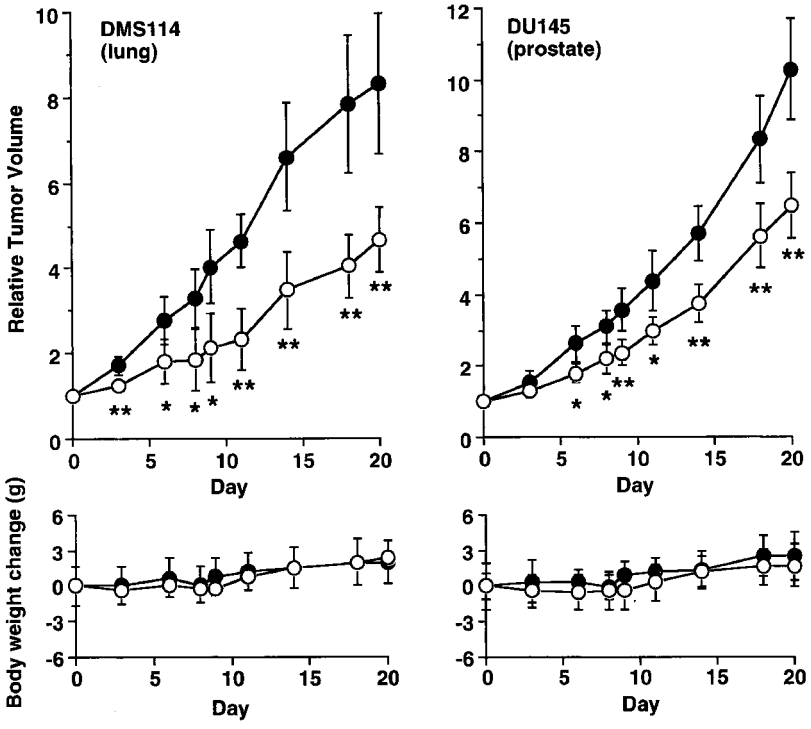

Fig. 5 Effect of BBGC on tumor growth and body weight change in nude mice bearing human cancer xenografts. Tumor inoculation was carried out as described in "Materials and Methods." Tumor-bearing mice were treated with BBGC daily from days 0 to 8 at doses of 0 (●, control) and 100 mg/kg (○). The data shown are means ± SD of five mice. Statistical evaluations were performed using the Student's *t* test, comparing the group receiving BBGC treatment with the group receiving control treatment. *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

The GLO1 expression is regulated under various physiological conditions and in disease processes. Elevated levels of GLO1 are associated with increased proliferative activity of the tumor (2, 3, 15). The occurrence of abnormally expressed GLO1 in tumor cells leads to the concept of GLO1 inhibitors as possible cancer therapeutic drugs. In this report, we found that GLO1 is specifically elevated in human lung cancer cells. We observed that the GLO1 inhibitor diester BBGC could be a potent therapeutic drug against human lung cancer, and that BBGC efficiently induced apoptosis in human lung cancer NCI-H522 and DMS114 cells (Fig. 2*B*). An important feature of BBGC-induced apoptosis is its specificity for cells expressing high GLO1 activity. Furthermore, a significant correlation was observed between cellular GLO1 enzyme activity and the sensitivity of human lung cancer cell lines to BBGC. These results indicate that BBGC could be a promising anticancer drug for GLO1-overexpressed lung cancer cells.

The precise mechanism of apoptosis induced by GLO1 inhibitors is still unknown. Inhibition of GLO1 led to the accumulation of MG in cells (5). Because MG is capable of inducing DNA modification and DNA-protein cross-link (16), DNA damage or inactivation of some antiapoptotic proteins by MG could be an initial activator of apoptosis. We have shown that activation of caspase-3 and -9 are essential for BBGC-induced apoptosis in NCI-H522 and DMS114 human lung cancer cells (Fig. 3 and data not shown). We and others have reported that a variety of cytotoxic stresses, such as DNA-damaging agents and ionizing radiation, caused activation of p38 and JNK, members of the MAPK superfamily, that were recently shown to be involved in stress-induced caspase activation (11, 17–20). In this study, we found that the GLO1 inhibitor, BBGC, induced both p38 and JNK activation prior to caspase activation in NCI-H522 cells. However, these activations were not observed in A549 cells, which were resistant to BBGC-induced apoptosis (Fig. 4). These results suggest that activation of p38 and JNK could be involved in BBGC-induced apoptosis through activation of downstream caspase cascades pathways.

We found that GLO1 inhibitor, BBGC, preferentially suppressed growth of cells that expressed higher GLO1 activity. We speculated that some tumor cells need higher levels of GLO1 because they could have elevated glycolytic activity and produce higher levels of MG in cells. Our data suggest that cells with high GLO1 activity have high MG-productive capacities, and treatment with BBGC produces a large quantity of MG and selectively leads to cell death.

We have established a human cancer cell line panel coupled with a drug sensitivity database (21, 22). We compared several standard anticancer drugs with BBGC for the growth inhibitory patterns using COMPARE analysis (23, 24). We found that the pattern of drug sensitivity to BBGC differed from those of various antitumor drugs, such as DNA topoisomerase inhibitors, alkylating agents and antimitotic agents (data not shown). These results suggest that GLO1 inhibitor could be an anticancer drug with a novel mode of action.

In the present study, we evaluated the antitumor activity of BBGC *in vivo* using human cancer xenografts. We obtained promising results with DMS114 and DU-145 (Fig. 5). The most remarkable feature of BBGC was its low toxicity. As shown in Fig. 5, the animals treated with BBGC were able to maintain their body weights. We compared tumor tissues with normal tissues for GLO1 expressions using 48 Dot Human Tumor/Normal Tissue Total RNA Dot Blot (BioChain Institute, Inc.).

As a result, we found that the *GLO1* gene was overexpressed in 16 of 24 tumor tissues compared with normal tissues (data not shown). That BBGC was shown to be less toxic to cells with lower levels of GLO1 could mean it had minimal toxicity *in vivo* against normal cells with lower GLO1 expression.

BBGC can have the pharmacological effects *in vivo*. At present, we do not have data for the effect of drug-metabolizing enzymes on BBGC *in vivo*. We speculated that, at least, the cyclopentyl ester groups of BBGC could be stable, because it is necessary for BBGC to penetrate into cells and BBGC repressed the growth of GLO1-overexpressed tumors *in vivo*. Additional studies are needed to evaluate the *in vivo* pharmacokinetic properties of BBGC.

In conclusion, we found that GLO1 was frequently elevated in human lung carcinoma cells, and that the GLO1 inhibitor BBGC selectively induced apoptosis in the cells expressing higher GLO1 activity. Our results showed that GLO1 inhibitor was an effective chemotherapeutic agent, with minimal side effect, against solid tumors that overexpressed GLO1. Additional studies are needed to perform combinational chemotherapy with BBGC and various antitumor agents *in vivo*.

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