

Full Length Research Paper

# Selective cytotoxicity of *Carthamus tinctorius* against glucose-deprived HT-29 human colon carcinoma cells

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**Glucose deprivation, a feature of poorly vascularized solid tumors, activates the unfolded protein response (UPR) which is a stress-signaling pathway in tumor cells that is associated with the molecular chaperone, glucose-regulated protein 78 (GRP78). Induction of GRP78 protects cells against programmed cell death. Methanolic extract of *Carthamus tinctorius* (CTE) induced selective cytotoxicity in glucose-deprived HT-29 human colon carcinoma cells; this effect was not seen under normal growth conditions. CTE also suppressed the accumulation of the GRP78 protein and was highly toxic in HT-29 cells, with  $IC_{50}$  values for colony formation of  $< 50 \mu\text{g/ml}$  under 2-deoxyglucose (2DG) supplemented and glucose-deprived conditions. Interestingly, apoptotic activity of CTE was also detected by Hoechst staining and flow cytometric analysis. Therefore, these results suggest that CTE prevent the up-regulation of GRP78 and exhibit selective cytotoxicity in glucose-deprived HT-29 cells.**

**Key words:** Glucose deprivation, unfolded protein response, (glucose-regulated protein 78) GRP78, *Carthamus tinctorius*, HT-29 cells.

## INTRODUCTION

Solid tumors are especially difficult to treat because of their incomplete vascularization (Lee et al., 2007). A defining characteristic of solid tumors is their capacity to divide aggressively and metastasize under conditions of nutrient and glucose deprivation, which is uncommon in normal tissues (Acker and Plate, 2002; Szegezdi et al., 2006; Hwang et al., 2007). Glucose deprivation is a physiological cell condition associated with several human diseases, including tissue ischemia and cancer (Semenza, 2003), and can disrupt protein folding in the endoplasmic reticulum (ER) (Harding et al., 2002). The ER is an organelle responsible for the synthesis, initial post-translational modification, folding, export, and secretion of membrane proteins. As such, ER homeostasis is critical for the survival of eukaryotic cells. The accumulation of unfolded proteins in the ER activates the unfolded protein response (UPR), which enhances cell survival by limiting the accumulation of unfolded or misfolded proteins in the ER (Park et al., 2004). Furthermore, UPR is induced by glucose

deprivation and expression of the ER-resident molecular chaperone, glucose-regulated protein (GRP) 78 (Hwang et al., 2008). GRP78 also referred to as immunoglobulin heavy chain binding protein (Bip), is considered a central regulator of ER function because of its role in protein folding and assembly. GRP78 induction is widely used as a marker of ER stress and the onset of UPR (Park et al., 2007). Owing to its antiapoptotic properties, stress-induced increase in GRP78 expression represents an important pro-survival component of the evolutionarily conserved UPR (Philip et al., 2004). Several studies have shown that GRP78 plays a role in protecting tumor cells against intracellular-mediated cytotoxicity and against the toxic effects of anticancer agents (Suzuki et al., 2001). It is also possible that up-regulation of GRP78 expression in solid tumors protects tumor cells against various stress, including glucose deprivation, hypoxia and anticancer treatment (Park et al., 2002).

Thus, substances that directly down-regulate GRP78 expression might be potential therapeutic agents for solid tumors, which are major obstacles to successful cancer chemotherapy (Gazit et al., 1999). In the course of our screening program for down-regulators of the GRP78, we employed the MTT reduction, LDH release, and colony formation assay. GRP78 can be induced by ER stress

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such as treatment with 2-deoxyglucose (2DG), which causes chemical stress-induced glucose deprivation (Ryoo et al., 2006; Choo et al., 2005). Using this screening system, we recently screened 435 varieties of herbal medicines and determined that *Carthamus tinctorius* (CT) exhibits a striking selective cytotoxicity in glucose-deprived HT-29 human colon carcinoma cells.

Here, we investigated the effect of CT on GRP78 activation and the role of apoptosis in the selective cytotoxicity of CT in glucose-deprived HT-29 cells.

## MATERIALS AND METHODS

*C. tinctorius* (CT) was kindly supplied by Kumkang Pharm Co., Changwon, Korea. To obtain the CT extract, 10 g of CT was mixed with 100 ml of methanol for 3 days at room temperature and then filtered through a Whatman No. 1 filter paper (Advantec, Tokyo, Japan). The methanol was then evaporated, and the dried methanolic extract was obtained. Hereafter, for simplicity, the methanolic CT extract is denoted as CTE. CTE was then dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/ml for experiments, and CTE was added to the cell culture medium such that the DMSO made up < 0.5% of the total volume of the culture. 2-deoxyglucose (2DG) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in sterilized distilled water to obtain a stock concentration of 2 M. Other organic chemicals were of analytical grade or complied with the established standards for cell culture experiments.

### Cell culture and treatments

HT-29 human colon carcinoma cells were obtained from Korean Cell Line Bank (KCLB). The cells were maintained in RPMI (Invitrogen, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mg/ml NaHCO<sub>3</sub> and were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. To induce the glucose-deprivation conditions, 2DG was added to the culture medium at a final concentrations 20 mM or glucose-free RPMI1640 medium (Invitrogen) was supplemented with 10% heat-inactivated FBS as described previously (Ogiso et al., 2000).

### MTT reduction assay

Cell viability was measured with blue formazan that was metabolized from colorless 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) by mitochondrial dehydrogenases, which are active only in live cells. HT-29 cells were preincubated in 96-well plates at a density of  $1 \times 10^5$  cells/ml for 24 h. Cells treated with various concentrations of CTE were treated with or without 2DG for 24 h. After incubation, the MTT reagent (5 mg/ml) was added to each of the wells and the plate was incubated for an additional 2 h at 37°C. The intracellular formazan product was dissolved in 100 µl of DMSO. The absorbency of each well was then measured at 540 nm using an ELISA reader (Model 680, BioRad, Hercules, CA, USA), and the percentage viability was calculated.

### Lactate dehydrogenase release assay

Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH). A LDH release assay kit was purchased from Wako Pure Chemical Industries (Osaka, Japan). HT-29 cells

were pretreated with various concentrations of CTE. After incubating for 30 min, the cells were treated with 20 mM 2DG for 24 h. The LDH release assay reaction was initiated in a 96-well plate by mixing 50 µl of cell-free supernatant with a potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate in a final volume of 100 µl. A colorimetric assay was performed, in which the amount of formazan salt was proportional to the level of LDH activity in the sample. The intensity of the resultant red color measured at 540 nm was therefore proportional to the LDH activity.

### Colony formation assay

For the colony formation assay, cells were seeded at  $1 \times 10^5$  cells/well in 24-well plates, cultured overnight, and treated with various concentrations of CTE in the presence of 2DG (20 mM) or in glucose-free medium for 24 h. The cells were then diluted in a CTE-deficient fresh medium, reseeded at  $1 \times 10^3$  cells/well in 6-well plates, and cultured under normal growth conditions for 7 to 8 days to allow the formation of colonies. The formed colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted (Ogiso et al., 2000).

### Observation of morphological changes

HT-29 cells were seeded at  $2 \times 10^5$  cells/well in 6-well plates and incubated overnight in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. The cells were pretreated with 100 µg/ml of CTE. After incubation for 30 min, the cells were treated with 20 mM 2DG for 24 h. The cellular morphology was observed using phase-contrast microscopy (Nikon, Tokyo, Japan).

### Western blot analysis

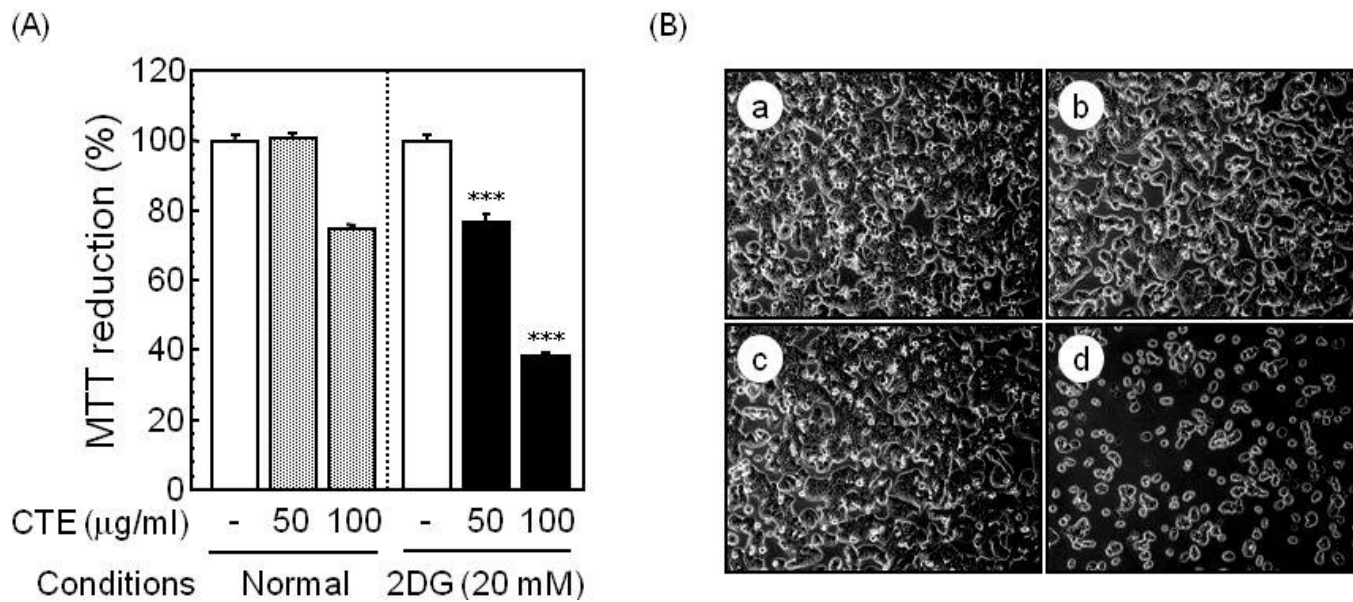
Whole cell lysates were prepared by solubilizing cells in 1 × sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol), as described (Ogiso et al., 2000). Proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane (BioRad) for western blotting. Membranes were probed with a mouse monoclonal anti-KDEL (for detection of GRP78, StressGen, Victoria, BC, Canada). Anti-mouse IgG HRP (Amersham Pharmacia Biotech, Tokyo, Japan) was used as the secondary antibody. Protein loading was controlled by probing the membranes for β-actin (Sigma-Aldrich) protein. Western blots were developed using the enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

### Cell staining

The morphology of apoptotic cells was investigated by staining the cells with Hoechst 33342 (Sigma-Aldrich) and visualizing them under a confocal spectral microscope (fluorescence microscopy). HT-29 cells ( $2 \times 10^5$  cells/ml) were plated in 6-well plates and treated with 100 µg/ml CTE under glucose-deprived conditions for 24 h. The cells were then washed with phosphate-buffered saline (PBS) and fixed in PBS containing 10% formaldehyde for 4 h at room temperature. After rinsing the cells twice with PBS, they were stained with Hoechst 33342 for 30 min at room temperature. The cells were again washed twice with PBS, and the Hoechst-stained nuclei were visualized under a fluorescence microscope (Nikon).

### Measurement of apoptotic cells

Flow cytometric analysis of cellular DNA content was performed as



**Figure 1.** Selective cytotoxicity of CTE in glucose-deprived HT-29 cells. (A) Cell viability was measured with the MTT reduction assay. HT-29 cells were exposed to an indicated concentration CTE for 24 h in the presence or absence of 2DG (20 mM). After the MTT reduction assay, MTT reduction rate was calculated by setting each of control survivals. (B) HT-29 cells were exposed to CTE for 24 h in the presence of 2DG (a; normal conditions, b; normal conditions/100 µg/ml CTE, c; 20 mM 2DG, d; 20 mM 2DG/100 µg/ml CTE). Photographs were taken using a phase-contrast microscope at 100× magnification. \*\*\*:  $p < 0.001$  compared with 2DG control.

described previously (Ogiso et al., 2000). Briefly, HT-29 cells were harvested and fixed with ice-cold 70% ethanol. The fixed cells were stained with 50 µg/ml propidium iodide at room temperature in the dark for 30 min. Apoptotic cells (mean values with 95% confidence intervals from triplicate determinations) were measured using a FACS caliber flow cytometer (Becton Dickinson).

#### Statistical analysis

All data are the means of 3 determinations. The data were analyzed using the SPSS package for Windows (Version 11.5; Chicago, IL, USA). The data were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's test.

## RESULTS AND DISCUSSION

### CTE induced selective cytotoxicity in glucose-deprived HT-29 cells

We screened 435 herbal medicine extracts, and have recently isolated an active substance in the CTE that preferentially exerts cytotoxic activity in glucose-deprived HT-29 cells. We examined the effects of CTE on cell viability by the MTT reduction assay. Under normal growth conditions, 24 h treatment of HT-29 cells with CTE had little effect on cell viability (Figure 1A). In contrast, cells exposed to CTE at 100 µg/ml showed a 50% reduction in cell viability as compared with the 2DG-stress controls. The morphological alterations of these cells were assessed by phase-contrast microscopy, after

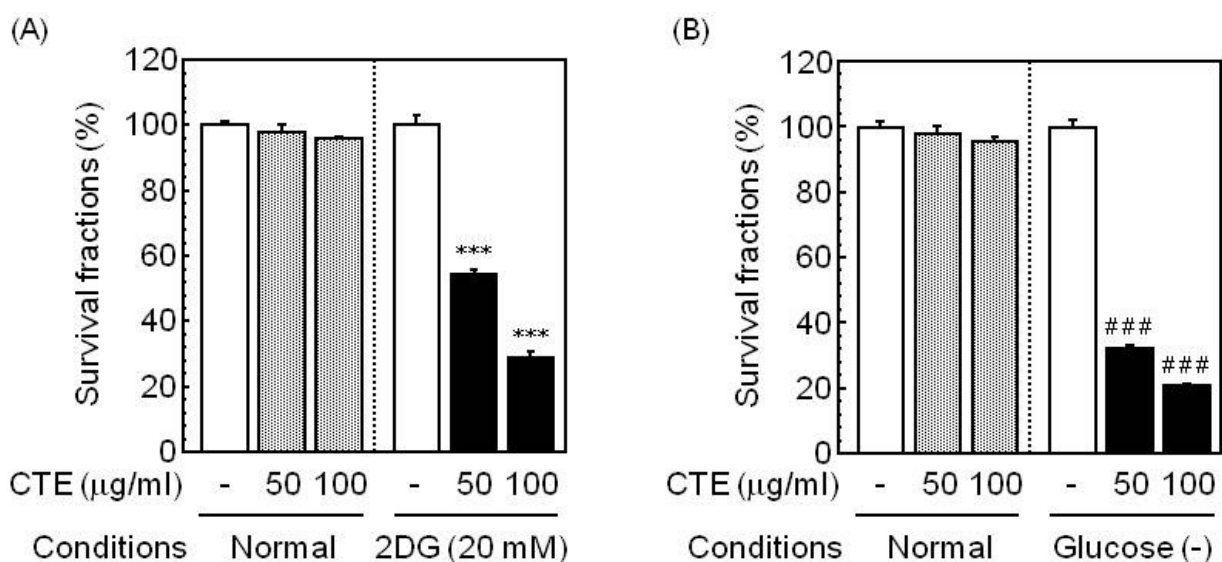
24 h of incubation with 100 µg/ml of CTE in the presence or absence 2DG (Figure 1B). During normal growth conditions, CTE exhibited no cytotoxic effects on cell morphology. However, CTE-treated cells exposed to 2DG-stress conditions exhibited cytoplasmic shrinkage, and either detached from each other or floated in the medium.

Next, to assess the selective cytotoxicity induced by CTE in HT-29 cells under glucose-deprived conditions, cells were incubated with CTE as described earlier and cytotoxicity was determined by LDH release and colony formation assays (Table 1 and Figure 2). The cytotoxic effect of CTE on HT-29 cells was evaluated by measuring the extent of LDH leakage into the medium (Table 1). The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage (Zhu et al., 2006). The HT-29 cells were exposed to 50 or 100 µg/ml of CTE under normal growth and 2DG-stress conditions for 24 h. As expected, under normal growth conditions, CTE little effect on HT-29 cell viability. In contrast, CTE-induced cell death occurred in a dose-dependent manner, as evidenced by 10 to 14% increase in LDH release in 2DG-stressed HT-29 cells. We further conducted a colony formation assay. In this assay, the CTE treatment was highly toxic to cells in the 2DG-containing or glucose-free medium, with a half-maximal inhibitory concentration ( $IC_{50}$ ) of < 50 µg/ml CTE (Figure 2A and B). These results suggest that CTE is not cytotoxic to HT-29 cells under normal conditions, but it preferentially reduces colon

**Table 1.** Selective cytotoxicity of CTE in glucose-deprived HT-29 cells.

Treatment	Concentration ( $\mu\text{g/ml}$ )	LDH release (%)
Control	-	18.0 $\pm$ 0.600
	50	17.6 $\pm$ 0.403
	100	18.3 $\pm$ 0.009
2DG (20 mM)	-	21.7 $\pm$ 0.659
	50	27.2 $\pm$ 0.557
	100	32.2 $\pm$ 0.463

Cytotoxicity of CTE was determined by the LDH release assay in the presence or absence of 2DG (20 mM). In the LDH release assay, data were normalized to the activity of LDH released from the vehicle-treated cells (100%) and expressed as percentage of control.



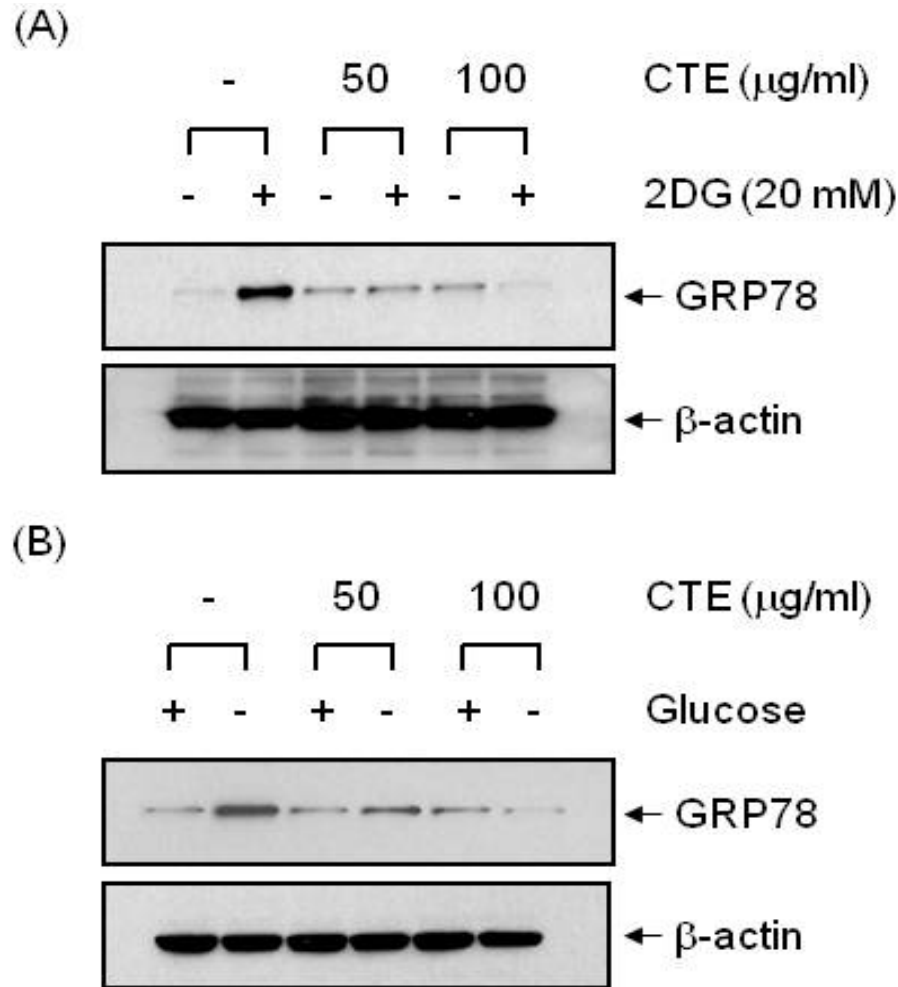
**Figure 2.** Selective cytotoxicity of CTE in glucose-deprived HT-29 cells. Colony formation analysis of HT-29 cells exposed to the indicated concentrations of CTE for 24 h in the presence or absence of 2DG (20 mM) (A) and glucose (B). After colony formation, the survival rate was calculated relative to each control survival rate. \*\*\*:  $p < 0.001$  compared with 2DG control. ###:  $p < 0.001$  compared with glucose deprivation control.

cancer cell viability under glucose-deprivation conditions. This implies that CTE may inhibit a response to glucose-deprived stress conditions in colon cancer cells and that CTE may be a potential candidate for a novel therapeutic approach.

#### Suppression of GRP78 expression and apoptotic response of CTE in glucose-deprived HT-29 cells

To examine a possible correlation between CTE cell-mediated selective cytotoxicity and GRP78-inhibiting activity, we performed a western blot analysis of HT-29 cells that had been subjected to glucose deprivation for 24 h in the presence or absence of CTE. As expected, CTE suppressed the expression of GRP78 protein under

2DG-stress or glucose-free conditions in glucose-deprived HT-29 cells but had no effect on the expression level of GRP78 under normal growth conditions (Figure 3A and B). To investigate the mechanism of cell death induced by CTE under glucose-deprived conditions, we examined the apoptotic activity in HT-29 cells following CTE treatment under glucose-free conditions by using Hoechst 33342 staining. We collected cells after CTE treatment in the presence or absence of glucose and examined the morphological changes in their nuclei. HT-29 cells treated with 100  $\mu\text{g/ml}$  of CTE for 24 h showed marked chromatin condensation and the formation of apoptotic bodies under glucose-deprived conditions, but not under normal growth conditions, indicating selective apoptosis in the CTE-treated glucose-deprived HT-29 cells (Figure 4). We also assessed whether cells were



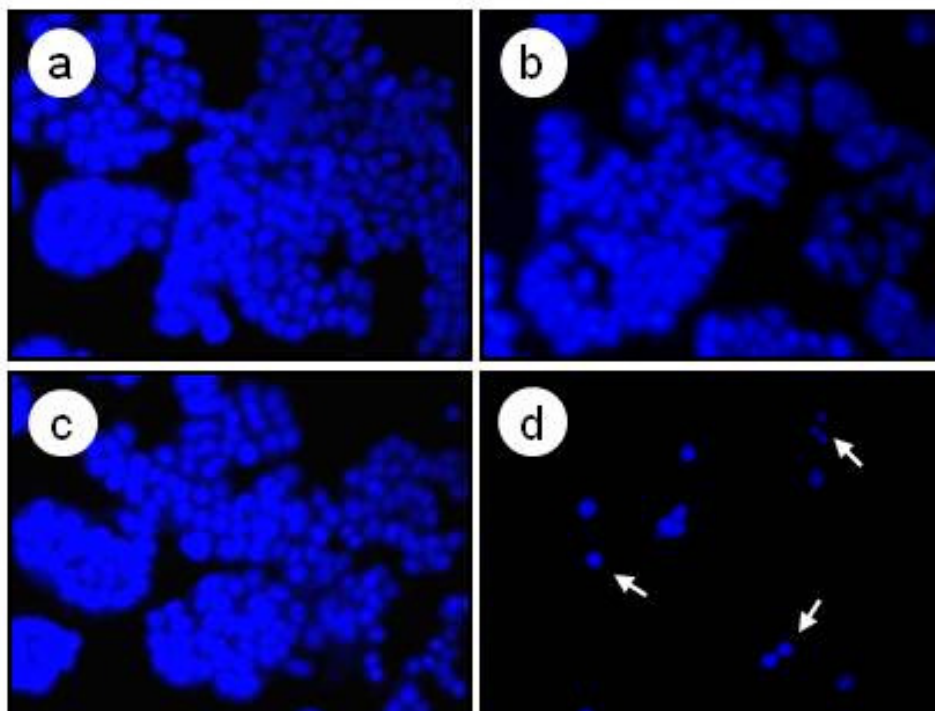
**Figure 3.** CTE-induced suppression of 2DG and glucose deprivation-induced GRP78 protein expression. Total cell lysates of HT-29 cells were prepared and subjected to western blot analysis by using mouse monoclonal anti-KDEL antibody for the detection of GRP78. HT-29 cells were treated with CTE at the indicated concentration for 24 h in the presence or absence of 2DG (20 mM) (A) and glucose (B).

similarly sensitized to glucose-deprived conditions by CTE treatment by using a flow cytometry assay of apoptotic cells that were distributed according to the cell cycle stage and showing sub-G1 DNA content. The number of apoptotic cells in the sub-G1 phase increased approximately 6-fold in the presence of 100  $\mu\text{g/ml}$  of CTE when compared with that in the case of control cells (Table 2). The data also indicated a depletion of cells in the G1 phase and a concomitant accumulation of cells in the G2/M phase in the glucose-deprived conditions. The arrest in the G2/M phase was accompanied by an increase in the number of sub-G1 cells, which is typical of the late stages of apoptosis. Thus, CTE-induced selective apoptosis in glucose-deprived HT-29 cells, and these antiproliferative outcomes were not present under normal glucose conditions.

These results indicate that the selective cytotoxicity of

CTE in glucose-deprived conditions correlates with the GRP78-inhibiting effect and that GRP78 inhibitory activity of CTE induces selective apoptosis in glucose-deprived HT-29 cells. Cancer cells are routinely under several physiological stress conditions such as glucose starvation, hypoxia and low pH. However, despite these stress conditions, cancer cells grow well by over-expression of GRP78, which serve as the survival factor (Moenner et al., 2007). Therefore, GRP78 is a crucial target for destroying cancer cells under physiological stress conditions. In an earlier study, we reported a novel macrocyclic compound, versipelostatin, which can specifically suppress GRP78 expression in response to glucose deprivation in cancer cells (Park et al., 2004).

From the same point of view, because CTE exhibited selective cytotoxicity in glucose-deprived colon cancer cells by blocking GRP78 expression, CTE may be a



**Figure 4.** CTE induced apoptosis of glucose-deprived HT-29 cells. Apoptotic nuclei were seen after CTE treatment for 24 h under glucose-deprived conditions (a; normal conditions, b; normal conditions/100 µg/ml CTE, c; glucose deprivation, d; glucose deprivation/100 µg/ml CTE). Fixed cells were stained with Hoechst 33342 and examined under a by fluorescence microscope. Photographs were taken using a blue filter at 400× magnification. Arrows indicate an apoptotic cell with apoptotic bodies.

**Table 2.** CTE-induced apoptosis in glucose-deprived HT-29 cells.

Treatment	Concentration (µg/ml)	Apoptotic cells (%)
Control	-	5.9±0.827
	100	23.0±6.505
Glucose (-)	-	7.5±0.764
	100	37.1±2.828

Flow cytometric analysis of apoptosis in HT-29 cells exposed to CTE with or without glucose. Sub-G1 events and apoptotic cells were measured by FACS analysis after propidium iodide staining.

potential candidate in cancer chemotherapy.

## Conclusion

This study clearly indicates that CTE selectively induces cytotoxicity in glucose-deprived HT-29 cells, possibly by blocking GRP78. Suppression of the GRP78 expression during glucose deprivation appeared to lead to reduced cell survival and increased apoptosis. Thus, CTE may be an attractive tool and should be explored for its potential use in glucose deprivation-targeted cancer therapy.

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