Selective Cytotoxic Activity of Valinomycin against HT-29 Human Colon Carcinoma Cells *via* Down-Regulation of GRP78

In-Ja Ryoo,[a] Hae-Ryong Park,[b] Soo-Jin Choo,[a] Ji-Hwan Hwang,[b] Young-Min Park,[c] Ki-Hwan Bae,[c] Kazuo Shin-Ya,[d,e] and Ick-Dong Yoo*,[a]

[a] *Laboratory of Antioxidant, Korea Research Institute of Bioscience and Biotechnology; Daejeon 305–333, Korea:* [b] *Division of Food Science and Biotechnology, Kyungnam University; Masan 631–701, Korea:* [c] *College of Pharmacy, Chungnam National University; Daejeon 305–764, Korea:* [d] *Institute of Molecular and Cellular Bioscience, The University of Tokyo; Bunkyo-ku, Tokyo 113–0032, Japan: and* [e] *BRIC, AIST, Protein Network Team, Low M.W. Chemical Laboratory; 2–42 Aomi, Koto-ku, Tokyo 135–0064, Japan.* Received September 20, 2005; accepted December 26, 2005

Glucose deprivation is a fundamental feature of poorly vascularized solid tumors and leads to activation of the molecular chaperone GRP78, which is associated with the unfolded protein response (UPR), a stress-signaling pathway, in tumor cells. We recently isolated an active compound, M126, that inhibits transcription from a GRP78 promoter reporter construct. M126 was identified as valinomycin by various spectroscopic methods. We found that valinomycin prevents UPR-induced protein expression, such as GRP78 and GRP94. The GRPs-inhibitory action of valinomycin severe hypoglycemic and results in selective cell death of the stressed cancer cells. Our findings demonstrate that GRP78 may be an excellent target for the use of cancer chemotherapy in the treatment of solid tumors.

Key words valinomycin; GRP78; HT-29; endoplasmic reticulum stress; unfolded protein response

Cancer cells in poorly vascularized solid tumors, *in vivo*, are often surrounded by stressful microenvironments, including glucose starvation, hypoxia, low pH and other nutrient deprivations which are not commonly observed in normal tissues.[1,2] These microenvironmental conditions, especially glucose deprivation, can disrupt protein folding in the endoplasmic reticulum (ER).[3—5] The accumulation of unfolded proteins in the ER activates the unfolded protein response (UPR), which enhances cell survival by induction of the molecular chaperones, glucose-regulated protein (GRP) 78 (also known as Bip) or GRP94. These GRPs are associate with nascent proteins, facilitating their translocation into the ER and aiding in their folding and transport through the ER. Several reports suggest that GRP78 may protect the host cells against cell death by suppressing oxygen radical accumulation and stabilizing mitochondrial function.[6—9]

The protective function of GRPs suggests that their induction could be beneficial in situations involving normal tissues or organ damages.[10] However, the anti-apoptotic function of GRPs could lead to cancer progression and drug resistance in neoplastic cells.[10—12] In a variety of cancer cell lines, solid tumors and human cancer biopsies, the levels of GRP78 and GRP94 are elevated and seem to correlate with malignancy.[13—15] In addition, induction of GRP78 has been shown to protect cancer cells from immune surveillance, whereas suppression of the stress-mediated induction of GRP78 enhanced apoptosis, inhibited tumor growth and increased the cytotoxicity of chronically hypoxic cells.[16—19] Thus, substances that directly down-regulate GRP78 might be expected to be use as drugs for the treatment of cancer.

In the course of our screening program for down-regulators of GRP78, we employed the reporter gene assay system utilizing the luciferase gene. HT1080 cells, which are transformed with the luciferase gene under the control of the *grp*78 promoter and thus designated as HT1080-GL cells, respond sensitively to luciferase *grp*78 induction by ER stress, such as treatment with 2-dexoyglucose (2DG) or tunicamycin (TM).[20] By using this screening system, we isolated compound M126 from the culture broth of actinomycete M020126 as a down-regulator of GRP78. By examining its structure and physico-chemical properties, its chemical structure was identified as that of valinomycin, which has been previously reported as a potassium ionophore.[21] However, GRP78 down-regulating activity of valinomycin has not been reported up to now. In this study, we describe the isolation, biological activity and GRP78 down-regulating activity of valinomycin.

MATERIALS AND METHODS

Microorganism and Culture Conditions Strain M020126 was inoculated into 500 ml Erlenmeyer flask containing 100 ml of GSM medium consisting of 1% soluble starch, 2% glucose, 0.5% molasses, 0.5% yeast extract, 0.5% peptone and 0.2% $CaCO_3$ adjusted to pH 7.2 and incubated at 28 °C for 7 d on a rotary shaker at 140 rpm.

Cell Treatments HT1080 fibrocarcinoma cells and HT-29 human colon carcinoma cells were maintained in RPMI1640 medium (Gibco) supplemented with 10% FBS (Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml) and 2 μg/ml $NaHCO_3$ at 37 °C in a humidified CO_2 incubator. 2-Deoxyglucose (2DG, Sigma) was added to the culture medium at the final concentrations 10 mM.

Construction of Plasmid and Transfection pGRP78pro160-Luc was created by cloning the human GRP78 promoter region (−160—+7; generated by PCR with 293T genomic DNA) into pGL3-Basic vector (Promega, Madison, WI, U.S.A.) at the *Kpn* I/*Hind* III site. We transfected pGRP78pro160-Luc into HT1080 cells using the FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) according to the manufacturer's protocol.

Luciferase Assay HT1080-GL cells were plated on 96-well microplate at a density of 1.5×10^4 cells/well with

* To whom correspondence should be addressed. e-mail: idyoo@kribb.re.kr

100 μl of medium. After incubation for 6 h, the cells were treated with test samples at various conditions for 18 h. Relative firefly-to-*Renilla* luciferase activity was measured using a Luciferase Reporter Assay system (Promega) according to the manufacture's instructions.

Cell Viability Assay The tetrazolium dye colorimetric test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test) was used to determine the viability of HT-29 human colon carcinoma cells.[22] HT-29 cells (1×10^5/well) were cultured in a 96-well microplate for 24 h in a humidified atmosphere of 5% CO_2 in air at 37 °C, and pretreated with different concentrations of test samples. After 30 min incubation, 2DG, a chemical stress, was added to the wells with a final concentration 10 mM, and the plates were reincubated. After 24 h of incubation, MTT reagent (5 mg/ml) was added to each of the wells, and the plate was incubated for an additional 4 h at 37 °C. The media were then removed, and the intracellular formazan product was dissolved in 100 μl of dimethyl sulfoxide (DMSO). The absorbency of each well was then measured at 540 nm, and the percentage viability was calculated.

Western Blot Analysis Cells extracted proteins were resolved by SDS-PAGE and transferred onto an Immunoblot PVDF membrane (Bio-rad) for Western blotting. Membranes were probed with mouse monoclonal anti-KDEL (for detection of GRP78 and GRP94, StressGen). Anti-mouse IgG HRP (Amersham Pharmacia Biotech) was used as a secondary antibody. Protein loading was controlled by probing the membranes for α-tubulin protein. Western blots were developed using the enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) according to the manufacture's instructions.

Cell Staining HT-29 cells were washed twice with phosphate-buffered saline (PBS) and then fixed in PBS containing 4% formaldehyde for 10 min at room temperature. Fixed cells were washed with PBS, and stained with 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc. Burlingame) solution for 10 min at room temperature. The cells were washed twice more with PBS and the DAPI-stained nuclei were visualized by using a fluorescence microscope.

RESULTS

Purification of GRP78 Down-Regulator Isolation and purification of GRP78 down-regulator was carried out from the culture broth of Actinomycete M020126. The culture broth was extracted with ethyl acetate three times and concentrated *in vacuo*. The crude extract was purified with silica gel TLC ($CHCl_3$: MeOH = 10 : 1). The active principle was applied to a Sephadex LH-20 column, which was developed with 100% MeOH. The structure of this active compound M126 was determined on the basis of ESI-MS and NMR experiments. The molecular formula of the M126 compound was established as $C_{54}H_{90}N_6O_{18}$ by ESI-MS spectroscopy (data not shown). Through a database and literature search, it was found that M126 had the same structure as valinomycin, which had been previously reported as a potassium ionophore.[21]

Effect of Valinomycin on the GRP78 Promoter Expression To investigate whether valinomycin has a direct effect on the human GRP78 promoter, we examined luciferase activity using HT1080-GL cells transfected with the reporter plasmid. This promoter region contains the *cis*-acting endoplasmic reticulum stress response element (ERSE), which is required for transcriptional activation in response to ER stress.[23] As shown in Fig. 1, in HT1080-GL cells, treatment with the hypoglycemia-mimicking agent 2DG increased firefly luciferase activity by approximately five-fold. Under 2DG stress, valinomycin strongly inhibited the luciferase activity mediated by 2DG with IC_{50} values of 10 nM. It also suppressed 2DG-inducible GRP78 promoter activity in a dose-dependent manner. Expectedly, under normal growth conditions, valinomycin had no effect on GRP promoter activity. These results clearly indicate that valinomycin selectively inhibits ERSE-dependent transcription during treatment with 2DG.

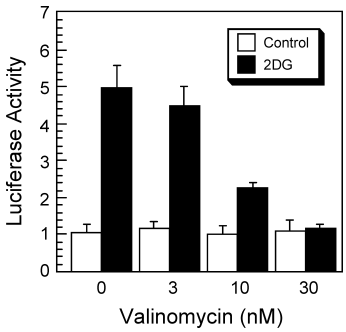

Fig. 1. Luciferase Reporter Assay to Express a Human GRP78 Promoter by Valinomycin in the Presence or Absence of 2DG

HT1080-GL cells were transfected with human GRP78 promoter and treated for 18 h with valinomycin under normal growth or 2DG stress conditions (10 mM) at the indicated concentrations. Data (means±S.D. of triplicate determinations) are representative of at least two independent experiments.

Effect of Valinomycin on the GRP78 Protein Expression To determine whether the observed decrease in GRP78 promoter correlated with decreased protein levels, we performed western blot analysis using HT-29 human colon carcinoma cells in the presence or absence of valinomycin. In time-course experiments, we found that valinomycin suppressed GRP78 and GRP94 induction in the hypoglycemia-mimicking agent 2DG-stressed HT-29 (Fig. 2A). Likewise, valinomycin also inhibits GRPs in glucose-starved HT-29, but did not affect the expression level under normal growth conditions (Fig. 2B). Valinomycin also suppressed 2DG- and glucose-starvation-induced GRPs expression in a dose-dependent manner (Figs. 3A, B). These results indicate that valinomycin selectively inhibits ER stress-dependent GRPs during glucose deprivation.

Effect of Valinomycin-Induced, Selective Cytotoxic Activity on Glucose-Deprived Cells We next examined the effects of valinomycin on cell viability by the MTT method. Under normal growth conditions, 24-h valinomycin treatment of HT-29 cells had only a general effect on cell viability. In contrast, the valinomycin treatment became highly toxic in the 2DG-containing medium under hypoglycemic conditions (Fig. 4A). Under the same conditions, cytotoxic activity correlated well with the inhibition of GRP78 and GRP94 expression. We evaluated the apoptotic activity of HT-29 cells to valinomycin treatment that was observed under 2DG conditions, as determined by DAPI stain assay of apoptotic cells showing nuclear condensation and fragmenta-

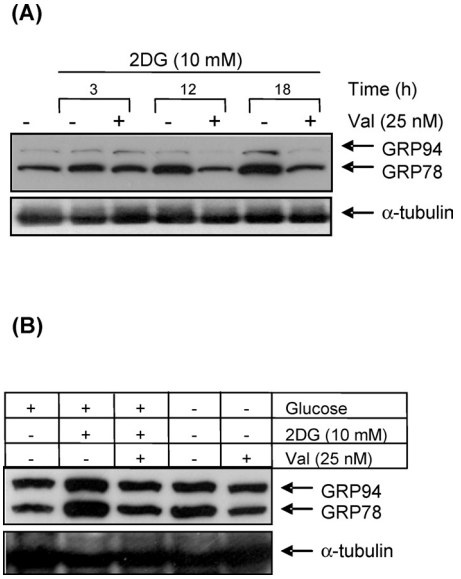

Fig. 2. Attenuated Expression of GRP78 and GRP94 Protein Levels in HT-29 Cells under 2DG Stress Condition or Glucose Starvation

Total cell lysates of HT-29 cells were prepared and subjected to Western blot analysis using mouse monoclonal anti-KDEL antibody. The cells were treated with 25 nM valinomycin (Val) for the indicated time periods (A) and under glucose deprivation condition (B) in the presence or absence of 2DG stress (10 mM). Data shown in all panels are representative of at least two independent experiments.

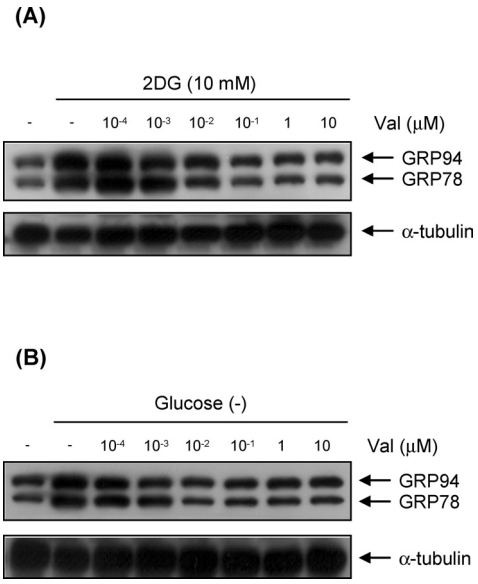

Fig. 3. Valinomycin Suppressed 2DG or Glucose Starvation-Induced GRP Proteins Expression in a Dose-Dependent Manner

Total cell lysates of HT-29 cells were analyzed by Western blot using anti-KDEL antibody. HT-29 cells were treated with valinomycin (Val) indicated concentration for 18 h under 2DG stress condition (10 mM) (A) and glucose starvation (B). Data shown in all panels are representative of at least two independent experiments.

tion. We collected cells after valinomycin treatment in the presence or absence of 2DG and examined the morphological changes of their nuclei. HT-29 cells treated with valinomycin for 18 h showed little apoptosis under normal growth conditions. In contrast, drastic apoptosis resulted from 2DG condition such as glucose being withheld for 18 h (Fig. 4B). Similarly, valinomycin treatment showed strong cytotoxicity of the 2DG-containing medium HT-29 cells in the MTT reduction assay. Taken together, valinomycin seemed to interfere with the proliferation and induction of apoptosis through

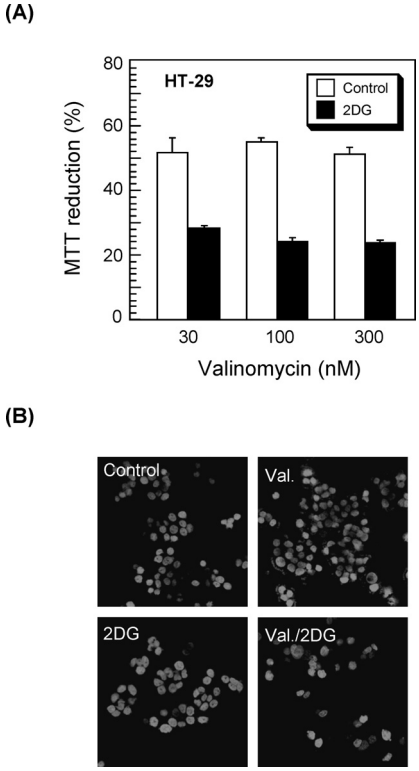

Fig. 4. Cytotoxic Activity and Apoptotic Nuclear Condensation Was Induced by Valinomycin under Stress Conditions

(A) HT-29 cells were exposed to an indicated concentrations valinomycin for 24 h in the presence or absence of 10 mM 2DG. After a MTT assay, the MTT reduction rate (means±S.D. of triplicate determinations) were calculated by setting each of control survivals without 2DG treatment as ER stress. (B) HT-29 cells were treated with valinomycin (10 nM) in the presence (lower panels) or absence (upper panels) of 2DG stressor (10 mM) for 18 h. The cells were collected, fixed, and stained with DAPI (1 μg/ml). Photographs were taken using blue filter at the magnification of ×100.

the inhibition of GRP78 and GRP94 in 2DG stress condition.

DISCUSSION

In our study, the GRP78 down-regulator, M126, was isolated from Actinomycete M020126 and identified as valinomycin, which has been previously reported as a potassium ionophore.[21] Valinomycin has often been used to dissipate the membrane potential of cells and mitochondria and to induce apoptosis.[24,25] A recent study reported that the effect of valinomycin as an apoptosis inducer was due to the release of mitochondrial cytochrome *c* in a permeability transition (PT)-independent manner.[26] However, there are no reports that valinomycin is associated with down-regulation of GRP78, a 78-kDa protein referred to as Bip.

As a molecular chaperone, GRP78 is known to form complexes with heterologous proteins that are processed through the ER.[2] As mentioned previously, GRP78 plays an important role in a protective mechanism for solid tumors.[3] Cancer cells proliferate by using GRP78 under micro-environmental conditions which occur naturally in solid tumors.[4,5] As this stress condition generally leads to the accumulation of misfolded proteins, induction of *grp*78 gene expression has been used extensively as a marker for the unfolded protein response (UPR).[23] Over-expression of the *grp*78 gene facilitates normalization of abnormal proteins, which may be protecting tumor cells *in vivo*.[10] Therefore, targeted suppression

of GRPs expression could be used as an approach to cancer chemotherapy.[27—29] Another GRP78 down-regulator, versipelostatin, was isolated from *Streptomyces versipellis*[20] and the effects of versipelostatin on a diversity of cell lines were studied, making clear the relationship between inhibitory activity against GRP78 expression and antitumor activity.[30]

In our current study, we have demonstrated that the effects of valinomycin on down-regulation of GRP78 induce selective cell death of cancer cell lines only under 2DG or glucose-starvation conditions. As shown in Fig. 1, stress-inducible GRP78 promoter activity increased luciferase activity by approximately five-fold, and this is suppressed by valinomycin. Expectedly, it had no effect on GRP78 promoter activity under normal growth conditions. Valinomycin also inhibited GRPs expression that is induced by glucose starvation. Consistent with its GRP78 promoter activity, valinomycin significantly decreased the 2DG-induced GRPs expression in a time- and dose-dependent manner.

These results clearly indicate that valinomycin reduces stress-induced GRP78 expression increased by 2DG. We provide strong new evidence that a novel therapeutic approach to induce selective cell death in solid tumor tissue culture in microenvironmental conditions involves the GRP78 down-regulating activity of valinomycin.

Acknowledgements This work was supported by a grant from National Research Laboratory Program funded by the Ministry of Science and Technology, Korea.

REFERENCES

1) Brown J. M., Giaccia A. J., *Cancer Res.*, **58**, 1408—1416 (1998).
2) Tomida A., Tsuruo T., *Anticancer Drug Design*, **14**, 169—177 (1999).
3) Ma Y., Hendershot L. M., *Cell*, **107**, 827—830 (2001).
4) Harding H. P., Calfon M., Urano F., Novoa I., Ron D., *Annu. Rev. Cell Dev. Biol.*, **18**, 575—599 (2002).
5) Kaufman R. J., Scheuner D., Schroder M., Shen X., Lee K., Liu C. Y., Arnold S. M., *Nat. Rev. Mol. Cell Biol.*, **3**, 411—421 (2002).
6) Guo H., Tittle T. V., Allen H., Maziiarz R. T., *Exp. Cell Res.*, **245**, 57—68 (1998).
7) Lee J. W., Annadora J., Bruce-Keller A. J., Kruman Y., Chan S. L., Mattson M. P., *J. Neurosci. Res.*, **57**, 48—61 (1999).
8) Liu H., Miller E., Water B., Stevens J. L., *J. Biol. Chem.*, **273**, 12858—12862 (1998).
9) Yu Z. F., Luo H., Fu W., Mattson M. P., *Exp. Neurol.*, **155**, 302—314 (1999).
10) Lee A. S., *Trends Biochem. Sci.*, **26**, 504—510 (2001).
11) Gosky D., Chatterjee S., *Biochem. Biophys. Res. Commun.*, **300**, 327—332 (2003).
12) Reddy R. K., Mao C., Baumeister P., Austin R. C., Kaufman R. J., Lee A. S., *J. Biol. Chem.*, **278**, 20915—20924 (2003).
13) Fernandez P. M., Tabbara S. O., Jacobs L. K., Manning F. C. R., Tsangaris T. N., Schwarts A. M., Kennedy K. A., Parierno S. R., *Breast Cancer Res. Treat.*, **59**, 15—26 (2000).
14) Gazit G., Lu J., Lee A. S., *Breast Cancer Res. Treat.*, **54**, 135—146 (1999).
15) Patierno S. R., Tuscano J. M., Kim K. S., Landolph J. R., Lee A. S., *Cancer Res.*, **47**, 6220—6224 (1987).
16) Hughes C. S., Shen J. W., Subjeck J. R., *Cancer Res.*, **49**, 4452—4454 (1989).
17) Jamora C., Dennert G., Lee A. S., *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 7690—7694 (1996).
18) Shen J. W., Hughes C. S., Chao C., Chai J., Bartels C., Gessner T., Subjeck J. R., *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 3278—3282 (1987).
19) Song M. S., Park Y. K., Lee J. H., Park K. S., *Cancer Res.*, **61**, 8322—8330 (2001).
20) Park H. R., Furihata K., Hayakawa Y., Shin-ya K., *Tetrahedron Lett.*, **43**, 6941—6945 (2002).
21) Pressman B. C., *Annu. Rev. Biochem.*, **45**, 501—530 (1976).
22) Hansen M. B., Nielsen S. E., Berg K., *J. Immunol. Methods*, **119**, 203—210 (1989).
23) Yoshida H., Haze K., Yanagi H., Yura T., Mori K., *J. Biol. Chem.*, **273**, 33741—33749 (1998).
24) Inai Y., Yabuki M., Kanno T., Akiyama J., Yasuda T., Utsumi K., *Cell Struct. Funct.*, **22**, 555—563 (1997).
25) Nomura Y., *Biol. Pharm. Bull.*, **27**, 961—963 (2004).
26) Shinohara Y., Almofti M. R., Yamamoto T., Ishida T., Kita F., Kanzaki H., Ohnishi M., Yamashita K., Shimizu S., Terada H., *Eur. J. Biochem.*, **269**, 5224—5230 (2002).
27) Choo S. J., Park H. R., Ryoo I. J., Kim J. P., Yun B. S., Kim C. J., Shin-ya K., Yoo I. D., *J. Antibiot.*, **58**, 210—213 (2005).
28) Umeda Y., Chijiwa S., Furihata Ka., Furihata Ke., Sakuda S., Nagasawa H., Watanabe H., Shin-ya K., *J. Antibiot.*, **58**, 206—209 (2005).
29) Kaneko M., Ninuma Y., Nomura Y., *Biol. Pharm. Bull.*, **26**, 931— 935 (2003).
30) Park H. R., Tomida A., Sato S., Tsukumo Y., Yun J., Yamori T., Hayakawa Y., Tsuruo T., Shin-ya K., *J. Natl. Can. Inst.*, **96**, 1300—1310 (2004).