Effect on Tumor Cells of Blocking Survival Response to Glucose Deprivation

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**Background:** Glucose deprivation, a feature of poorly vascularized solid tumors, activates the unfolded protein response (UPR), a stress-signaling pathway, in tumor cells. We recently isolated a novel macrocyclic compound, versipelostatin (VST), that inhibits transcription from the promoter of GRP78, a gene that is activated as part of the UPR. We examined the effect of VST on the UPR induced by glucose deprivation or other stressors and on tumor growth in vivo.

**Methods:** Human colon cancer HT-29, fibrosarcoma HT1080, and stomach cancer MKN74 cells were cultured in the absence of glucose or in the presence of glucose and a UPR-inducing chemical stressor (the N-glycosylation inhibitor tunicamycin, the calcium ionophore A23187, or the hypoglycemia-mimicking agent 2-deoxyglucose [2DG]). The effect of VST on UPR induction was determined by reverse transcription–polymerase chain reaction and immunoblot analysis of the UPR target genes GRP78 and GRP94; by immunoblot analysis of the UPR transcriptional activators ATF6, XBP1, and ATF4; and by analyzing reporter gene expression in cells transiently transfected with a GRP78 promoter–reporter gene. Cell sensitivity to VST was examined with a colony formation assay and flow cytometry. In vivo antitumor activity of VST was assessed with an MKN74 xenograft model.

**Results:** VST inhibited expression of UPR target genes in glucose-deprived or 2DG-treated cells but not in cells treated with tunicamycin or A23187. VST also inhibited the production of the UPR transcriptional activators XBP1 and ATF4 during glucose deprivation. The UPR-inhibitory action of VST was seen only in conditions of glucose deprivation and caused selective and massive killing of the glucose-deprived cells. VST alone and in combination with cisplatin statistically significantly (P = .004 and P < .001 for comparisons with untreated control, respectively) inhibited tumor growth of MKN74 xenografts. Conclusion: Disruption of the UPR may provide a novel therapeutic approach to targeting glucose-deprived solid tumors. [J Natl Cancer Inst 2004;96:1300–10]

Glucose deprivation is a physiological cell condition that is associated with several human diseases, including tissue ischemia and cancer (1). Cancer cells in poorly vascularized solid tumors are constantly or intermittently exposed to glucose deprivation as well as to hypoxia (2,3). Glucose deprivation can disrupt protein folding in the endoplasmic reticulum (ER) (4–6). The accumulation of unfolded proteins in the ER activates the unfolded protein response (UPR), which enhances cell survival by limiting accumulation of unfolded or misfolded proteins in the ER (5,6). Several genes are transcriptionally activated as part of the UPR, including the ER-resident molecular chaperones GRP78 (also known as BiP) and GRP94 (7). Attenuation of the UPR by gene targeting renders mouse embryonic fibroblasts vulnerable to glucose deprivation (8). In theory, therefore, the UPR could be exploited for the selective killing of glucose-deprived solid tumor cells.

The UPR is initiated through signaling of the ER-localized transmembrane proteins ATF6, IRE1α (and, in some cells, IRE1β), and PERK (4–6). These signaling pathways produce several different active transcription factors that lead in turn to the coordinated expression of multiple UPR target genes (4–6). For example, ATF6 undergoes proteolytic cleavage to become an active transcription factor for UPR target genes (9–11), whereas IRE1α mediates the unconventional splicing of XBP1 mRNA, resulting in a potent UPR transcriptional activator (12–15). PERK induces the expression of the transcription factor ATF4 by transiently inhibiting general protein synthesis (16–18).

In the course of a screen for modulators of molecular chaperones, we recently isolated a novel compound from Streptomyces versipellis, designated versipelostatin (VST), that has a novel 17-membered macrocyclic structure with an α-acytetyronic acid moiety (19). VST can inhibit transcription from a GRP78 promoter reporter construct (19). Here, we investigated the effect of VST on UPR activation in cells exposed to glucose deprivation or other UPR-inducing stressors by examining the expression of UPR target genes and their activators. We also examined the in vivo antitumor activity of VST.

**Materials and Methods**

**Chemicals**

VST was prepared as a stock solution of 10 mM in methanol or phosphate-buffered saline and stored at −20 °C (19). n-Glucose and 2-deoxyglucose (2DG) were purchased from Sigma (St. Louis, MO), dissolved in sterilized distilled water at stock concentrations of 200 mg/mL and 2 M, respectively, and stored at −20 °C. Tunicamycin and cycloheximide (CHX) were purchased from Nacalai Tesque (Kyoto, Japan), A23187 from Wako Pure Chemical Industries (Osaka, Japan), and MG132

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See “Notes” following “References.”

DOI: 10.1093/jnci/djh243

Journal of the National Cancer Institute, Vol. 96, No. 17, © Oxford University Press 2004, all rights reserved.
from the Peptide Institute (Osaka, Japan). These compounds were dissolved in dimethyl sulfoxide (DMSO) at stock concentrations of 4 mg/mL, 10 mg/mL (CHX), 10 mM (A23187), and 10 mM (MG132) and were stored at −20 °C. All of the compounds were added to cell culture medium such that the solvent (methanol or DMSO) made up less than 0.5% of the volume of the culture medium.

Cell Lines

Human HT-29 colon cancer, HT1080 fibrosarcoma, and MKN74 stomach cancer cells were obtained from Dr. R. Shoemaker of the National Cancer Institute (National Institutes of Health, Bethesda, MD), the American Type Culture Collection (Manassas, VA), and IBL (Gunma, Japan), respectively. These cell lines were maintained in RPMI 1640 medium (containing 2 mg of glucose/mL; Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/mL of kanamycin and were cultured at 37 °C in a humidified atmosphere containing 5% CO2 (defined as “normal growth conditions”). Glucose-free RPMI 1640 medium was obtained from Invitrogen (San Diego, CA) and supplemented with 10% heat-inactivated fetal bovine serum as described previously (20,21). All in vitro experiments were performed using exponentially growing cells and were repeated at least twice.

Cell Treatments

To induce the UPR, we treated cells for various times with glucose deprivation by replacing the medium with glucose-free medium or by adding to glucose-containing culture medium one of the chemical stressors 2DG (20 mM), tunicamycin (TM) (5 μg/mL), or A23187 (1 μM). VST was added to the cells at various final concentrations immediately after they were placed in glucose-free medium or just before the chemical stressors were added to glucose-containing culture medium. In experiments involving CHX, it was also added to the cells in the same way as VST. In experiments that involved heat treatment, culture plates were floated on the water in a 42 °C water bath for 1 hour immediately after VST and 2DG were added to glucose-containing culture medium. In some experiments, we treated cells with the proteasome inhibitor MG132 at 10 μM during exposure to the UPR inducers.

Semiquantitative RT–PCR Analysis

Total RNA was isolated from HT-29 and HT1080 cells by using an RNeasy Mini Kit with DNase digestion (Qiagen, Tokyo, Japan) and was converted to cDNA with SuperScript II reverse transcriptase (Invitrogen). The cDNAs for GRP78, GRP94, HSP70, XBP1, and G3PDH were then amplified from the Peptide Institute (Osaka, Japan). These compounds were dissolved in dimethyl sulfoxide (DMSO) at stock concentrations of 4 mg/mL, 10 mg/mL (CHX), 10 mM (A23187), and 10 mM (MG132) and were stored at −20 °C. All of the compounds were added to cell culture medium such that the solvent (methanol or DMSO) made up less than 0.5% of the volume of the culture medium.

Plasmids and Transfection

The pcFlag vector was produced by ligating an oligonucleotide DNA sequence encoding a FLAG epitope to the HindIII site of pcDNA3 (Invitrogen). The plasmids pATF6(F), which codes for full-length ATF6; pATF6(A), which corresponds to the active form of ATF6; i.e., amino acids 1–373; pXBP1(U), which encodes the XBP1 protein corresponding to the unspliced mRNA; and pXBP1(S), which encodes the XBP1 protein corresponding to the spliced mRNA were created by inserting each cDNA [generated by PCR from untreated or 2DG-treated HT-29 cDNA, using the following primer pairs (5′ to 3′): CATC CCGGTACCGGAGCCGCCTGGGTGTTG and TGCTCT AGAGTCACGCTTTCTCCAG for pATF6(F); CATC CAAACCTTTTGAGGGACCCTGTGGGTGTTG and TGCT CTAGTCAAATCTAGGCTACTAAGCCTG for pATF6(A); and CATCCCAAGCTTTTGCTGGTGCTGACGCGCGCC GAAC and TGCTCTAGAAGTCAAGGAAAAGGGCACAG for pXBP1(U) and pXBP1(S)] in frame into the pcFlag vector at the HindIII/XbaI site. The pGRP78pro160-Luc plasmid was created by cloning the human GRP78 promoter region [nucleotides −160 to +7 relative to the start of transcription; generated by PCR, using the primer pair (5′ to 3′) CGGGGTACCGGGAG CAGTGACGTTTATTGCG and CATCCCAAGCTTTTGCTGGAC ACCCGTCGCCACTC, from genomic DNA isolated from 293T cells with the QIAamp DNA Mini Kit (Qiagen)] into the KpnI/HindIII site of the pGL3-Basic vector (Promega, Madison, WI), which contains the firefly luciferase gene. pHSHP7pro-Luc was created by inserting the human HSP70 promoter fragment (1.4 kb; upstream of +114 with respect to the start of transcription site), obtained by BglII/HindIII digestion of the Mammalian Heat Shock Expression Vector p2500-CAT (StressGen), into the BglII/HindIII site of the pGL3-Basic vector. pcDNA3.1/myc-His/IaclZ was purchased from Invitrogen. The proper construction of all plasmids was confirmed by DNA sequencing. Transient transfections were performed using the FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s protocol.

Immunoblot Analysis

Cells were lysed in 1× sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl [pH 6.8], 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol), and protein concentrations of the lysates were measured with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) (20,21). Equal amounts of proteins were resolved on a 4%–20% SDS–polyacrylamide gel and transferred by electroblotting to a nitrocellulose membrane (20,21). Immunoblots were probed with the following primary antibodies: mouse monoclonal anti-KDEL (for detection of GRP78 and GRP94; StressGen, Victoria, British Columbia, Canada); anti-FLAG M2 (for detection of FLAG-tagged ATF6 and XBP1 proteins; Sigma); anti-Myc tag (for detection of Myc-tagged β-galactosidase; Sigma); anti–β-
actin (for internal control; Sigma); anti-ATF6 (Active Motif, Carlsbad, CA); and rabbit polyclonal anti-CREB-2 (ATF4) (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were pretreated for 1 hour with TBS–T (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk at room temperature and incubated for 1–4 hours with the above primary antibodies (1:500 for anti-ATF6 antibody and 1:1000 for the other antibodies) under the same conditions. The membranes were washed with TBS–T containing 5% nonfat dry milk at room temperature and incubated for 1 hour with horseradish peroxidase–conjugated sheep anti–mouse or donkey anti–rabbit immunoglobulin antibody (1:1000) (Amersham Pharmacia Biotech, Tokyo, Japan) under the same conditions. After washing with TBS–T, the specific signals were detected with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

**Reporter Gene Assay**

HT1080 cells (3 × 10^5 in each well of 12-well plates) were cultured overnight under normal growth conditions. The medium was then changed to antibiotic-free RPMI 1640 medium supplemented with 5% fetal bovine serum, transfection mixtures that contained 375 ng of the firefly luciferase gene–containing reporter plasmids pGRP78pro160-Luc, pHS7p70pro-Luc, or pGL3 along with 125 ng of plasmid pRL-TK (Promega) (in which Renilla luciferase expression is under the control of the herpes simplex virus thymidine kinase promoter) as an internal control were added, and the cells were incubated for 8 hours at 37 °C in a CO₂ incubator. In some experiments, the reporter plasmids were combined with 300 ng of plasmid mixtures containing pcFlag (mock transfection) with various amounts of pATF6(A), pATF6(F), or pXBPI(U). In the case of pXBPI(S) co-transfections, we used 1.7 ng of pRL-CMV (Promega) (in which Renilla luciferase expression is under the control of the cytomegalovirus promoter) as an internal control with 500 ng of pGRP78pro160-Luc. The medium was then replaced with fresh medium lacking plasmid DNA, and the cells were incubated under the same conditions for another 4 hours. The cells were then replated in 96-well plates (at 5 × 10^3 cells/well), cultured overnight, and treated for 18 hours with various concentrations of VST treatment (42 °C) was carried out during the first 60 minutes of VST treatment. Ratios of firefly luciferase activity to Renilla luciferase activity (mean values with 95% confidence intervals from triplicate determinations) were determined using the dual luciferase kit (Promega).

**Measurement of Protein Synthesis**

Protein synthesis was assayed by measuring incorporation of [3H]alanine into trichloroacetic acid (TCA)–insoluble material. Cells were seeded at 4 × 10^3 or 8 × 10^3 in each well of a 96-well plate, cultured overnight under normal growth conditions, and then treated with various concentrations of VST for various times. During the last 1 or 2 hours of VST treatment, 2 μCi/mL of l-[2,3,–3H]alanine (52 Ci/mmol; Amersham Pharmacia Biotech) was added to the cells. The cells were then recovered on glass filters (Molecular Devices, Tokyo, Japan) with a cell harvester (Molecular Devices), and 1 mL/well of 5% TCA was passed through the filters, which were then washed with ethanol. The radioactivity on each filter was measured by scintillation counting. For cell counting, duplicate plates were treated with VST in the absence of [3H]alanine, and then the culture medium was replaced with fresh medium lacking VST. The cells were further cultured for an additional 2 hours under normal growth conditions, and cell number was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay (20). The amount of radioactivity incorporated was normalized to cell number, and relative radioactivity (mean values with 95% confidence intervals from triplicate determinations) was calculated.

**Cell Viability Assays**

Cells were seeded at 10^5 or 2 × 10^5 in 12- or six-well plates, cultured overnight, and treated with various concentrations of VST for various times. For the colony formation assay, cells were then diluted in fresh medium lacking VST, reseeded at 8 × 10^3 cells per well in six-well plates, and cultured under normal growth conditions for 7–8 days to form colonies (20,21). Cell survival (mean values with 95% confidence intervals from triplicate determinations) was calculated by setting the survival of control cells (i.e., not treated with VST) as 100%. IC₅₀ values (concentration required for 50% inhibition of colony formation) were determined from dose–response curves of colony formation inhibition. For the flow cytometry assays, the cells were fixed with 70% ethanol, treated with RNase, and stained with propidium iodide (22) or were stained directly (without ethanol fixation) with 7-aminocoumarin D (PharMingen, San Diego, CA). Apoptotic and dead cells (mean values with 95% confidence intervals from triplicate determinations) were counted using a Beckman-Coulter flow cytometer.

The growth inhibition assay with the human cancer cell line panel and the COMPARE analysis were performed as described previously (23–25). In brief, the cell line panel consists of the following 39 human cancer cell lines: lung cancer lines NCI-H23, NCI-H226, NCI-H522, NCI-H460, A549, DMS273, and DMS114; colorectal cancer lines HCC-2998, KM-12, HT-29, HCT-15, and HCT-116; gastric cancer lines MKN-1, MKN-7, MKN-28, MKN-45, MKN-74, and St-4; ovarian cancer lines OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SK-OV-3; breast cancer lines BSY-1, HBC-4, HBC-5, MDA-MB-231, and MCF-7; renal cancer lines RXF-631L and ACHN; melanoma line LOXIMVI; glioma lines U251, SF-295, SF-539, SF-268, SNB-75, and SNB-78; and prostate cancer lines DU-145 and PC-3. All cell lines were cultured in RPMI 1640 supplemented with 5% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C in humified air containing 5% CO₂. The GI₅₀ (concentration required for 50% growth inhibition) of VST for each cell line was determined after 48 hours of drug treatment with a sulforhodamine B assay (23–25). We then used the COMPARE algorithm to compare the GI₅₀ of VST and each of more than 400 standard compounds (including various anticancer drugs and inhibitors of biological pathways) and assessed the correlations between the differential growth inhibition patterns of VST and each standard compound in the cell line panel by determining the Pearson correlation coefficient, as described previously (23, 25).
MKN74 Xenograft Tumors

MKN74 cells were grown as subcutaneous tumors in nude mice, and 3 x 3 x 3-mm tumor fragments were then inoculated subcutaneously into nude mice, as described (23). Therapeutic experiments (involving six mice per group) were started when tumors had grown to approximately 100 mm3 (day 0). VST was administered intravenously at 13.5 or 18 mg/kg of body weight per day on days 0, 1, and 2. The control group received phosphate-buffered saline. For combination treatment, cisplatin was administered intravenously at 7 mg/kg on day 2. The mice were weighed twice each week up to day 24 to monitor the toxic effects. Tumor volume was determined by measuring the length (L) and width (W) of each tumor twice a week, up to day 24, and was calculated as (L x W^2)/2 (23).

Statistical Analysis

The statistical significance levels of differences in xenograft volume between groups of control and treated mice were evaluated using a one-way analysis of variance with Dunnett’s test as described previously (21). Differences with P values less than .05 were deemed statistically significant using a two-tailed test between the groups of control and drug-treated mice.

RESULTS

VST and Response to Glucose Deprivation and Other UPR-Inducing Stressors

To examine the effect of VST on endogenous GRP78 gene expression, we carried out a semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis of human colon carcinoma HT-29 and fibrosarcoma HT1080 cells that had been subjected to glucose deprivation for 18 hours in the presence or absence of VST. Whereas VST suppressed induction of GRP78 and GRP94 mRNA in glucose-starved HT-29 and HT1080 cells in a concentration-dependent manner (Fig. 1, a and b), it had no effect on GRP78 and GRP94 expression levels in cells grown in the presence of glucose (Fig. 1, b). Immunoblot analysis of lysates from the glucose-starved cells showed that VST also suppressed accumulation of the GRP78 and GRP94 proteins (Fig. 1, d).

To investigate whether the effect of VST on GRP expression extended to divergent ER stress stimuli, we treated HT-29 and HT1080 cells with one of three different types of chemical stressors: the hypoglycemia-mimicking agent 2DG, the N-glycosylation inhibitor tunicamycin, and the calcium ionophore A23187. As expected, VST suppressed 2DG-induced GRP78 and GRP94 mRNA (Fig. 1, b) and protein (Fig. 1, d) accumulation at the same concentrations at which it suppressed their accumulation in glucose-deprived cells. Time-course experiments revealed that the inhibitory effect was seen from the onset of GRP mRNA induction and was maintained for up to 18 hours (Fig. 1, c, left). However, VST had no effect on GRP78 or GRP94 mRNA expression at any concentration or time point in cells that were stressed by treatment with TM (Fig. 1, b and c); it also had no effect on GRP78 and GRP94 protein accumulation in TM- or A23187-treated cells (Fig. 1, d).

We also examined the effect of VST on human GRP78 promoter activity in HT1080 cells transiently transfected with a mammalian reporter gene plasmid (pGRP78pro160-Luc) that
includes the -160 to +7 promoter region of GRP78 cloned immediately upstream of the firefly luciferase gene. This promoter region contains the cis-acting endoplasmic reticulum stress response element (ERSE), which is required for transcriptional activation in response to ER stress (26). Because global protein synthesis was affected by the UPR-inducing stimuli whether or not VST was present, we could not compare absolute firefly luciferase activities; instead, we co-transfected cells with a control plasmid that contained a Renilla luciferase gene and compared the ratios of the two luciferase activities. In HT1080 cells transfected with the pGPR78pro160-Luc plasmid, treatments with 2DG and TM increased relative firefly luciferase activity by approximately five- and sevenfold, respectively (Fig. 1, e). Consistent with the GRP78 mRNA analysis, VST suppressed 2DG-induced GRP78 promoter activity in a dose-dependent manner (Fig. 1, e, left) but had little effect on TM-induced GRP78 promoter activity, even at 30 μM, a concentration 10 times higher than that at which it suppressed 2DG-induced GRP78 promoter activity (Fig. 1, e, right). Under normal growth conditions, VST (1–30 μM) had no effect on GRP78 promoter activity. For use as a control, we transfected cells with the pGL3-Control vector, which contains simian virus 40 promoter and enhancer sequences driving firefly luciferase activity, and found that the promoter activity was not affected by VST alone, by each of chemical stressors 2DG and TM, or by combinations of the two compounds (Fig. 1, e).

To further define the specificity of the inhibitory effects of VST, we examined expression of HSP70 (HSPA1A, NM_005345), which represents a class of stress-inducible molecular chaperones distinct from those of the GRP family. HSP70 mRNA levels in HT1080 cells increased in response to glucose deprivation but decreased in response to 2DG and TM (Fig. 1, b and c). VST (at 3 or 10 μM) had no effect on the changes in HSP70 expression in the glucose-deprived and the TM-stressed cells. In the 2DG-stressed cells, VST at 10 μM strongly induced HSP70 gene expression. Thus, VST was incapable of inhibiting HSP70 gene expression under all conditions examined. We also determined the effect of VST on promoter activity of another HSP70 gene (HSPA6, NM_002155) by using HT1080 cells transfected with the mammalian reporter plasmid pHS70pro-Luc. Although HSP70 promoter activity was inducible by treatment with heat (42 °C, 60 minutes), with 2DG, and with both together, VST at 3 μM had little effect on promoter activity under any of these conditions (Fig. 1, f). Taken together, these results indicate that VST selectively inhibits ERSE-dependent transcription during glucose deprivation.
inhibited the accumulation of GRP78 mRNA (Fig. 2, b; compare Fig. 1, b). Inhibition of XBP1 mRNA splicing was also observed at all time points examined (4–18 hours, Fig. 2, c). In addition, VST treatment decreased the total expression levels of XBP1 mRNA under hypoglycemic conditions (Fig. 2, b and c), an observation that is consistent with the fact that XBP1 mRNA is induced by ER stress in an ERSE-dependent manner (27). Finally, VST had no effect on TM-induced splicing of XBP1 mRNA (Fig. 2, b and c), further demonstrating the selective action of VST on hypoglycemic cells.

Immunoblot analysis of proteins from HT1080 cells transfected with each expression plasmid [pXBP1(S), pXBP1(U), pATF6(A), or pcDNA3.1/myc-His/LacZ (as a control)] showed that levels of the spliced XBP1 protein decreased more rapidly than those of the other proteins when VST was added to cells stressed by 2DG treatment (Fig. 2, d). Thus, VST can also operate at the post-translational level, thereby leading to complete loss of spliced XBP1 protein. Levels of unspliced XBP1 protein, as well as of the active form of the ATF6 protein, decreased more gradually in the presence of VST under 2DG stress conditions (Fig. 2, d), possibly as a result of active repression of protein synthesis in response to ER stress (28). Indeed, we estimated protein synthetic activity by measuring incorporation of tritiated alanine into protein. We found that overall protein synthesis was repressed by VST alone and by 2DG alone but was repressed to a much greater extent by the combination of VST and 2DG (Fig. 3, a). Effect was seen over all time periods of treatment examined (4–30 hours). However, VST alone had much less effect on global protein synthesis than the translation elongation blocker cycloheximide, which strongly inhibited protein synthesis in both the presence and absence of 2DG (Fig. 3, b). VST also enhanced protein synthesis repression during glucose deprivation but not during TM-induced stress (Fig. 3, c). Thus, VST enhanced translational repression and prevented UPR activation in hypoglycemic cells specifically.

**Roles of XBP1 and ATF6 in VST Action**

To address whether the depletion of spliced XBP1 protein induced by VST is involved in the inhibition of ERSE-dependent transcription, we co-transfected HT1080 cells with the reporter plasmid pGRP78pro160-Luc and pXBP1(S). Co-transfection of a relatively small amount (3 ng) of pXBP1(S), which encodes the spliced form of XBP1, was enough to enhance the basal reporter activity (i.e., in the absence of 2DG), and the activity was further increased by 2DG treatment (Fig. 4, a, left). Under these experimental conditions, 10 μM VST was required to achieve complete inhibition of 2DG-induced reporter gene activity in the pXBP1(S)-transfected cells, whereas 3 μM was sufficient in mock-transfected cells. Thus, ectopic expression of the spliced form of XBP1 conferred resistance to the inhibitory activity of VST against the ERSE-dependent transcription. By contrast, co-transfection with even large amounts (300 ng) of a plasmid encoding the unspliced form [pXBP1(U)] had little effect on basal reporter gene activity or VST sensitivity (Fig. 4, b).

When the cells were transfected with 300 ng of pXBP1(S), basal reporter gene activity was strongly enhanced such that no further activity was induced by 2DG treatment (Fig. 4, a, right). Thus, excess spliced XBP1 was expressed under these experimental conditions. VST did not inhibit reporter gene expression under normal growth conditions in these cells, but it still inhibited the reporter gene activity in 2DG-stressed cells, probably due to its ability to deplete spliced XBP1 protein under these stress conditions (Fig. 2, d). Collectively, these results suggest that the ability of VST to deplete the spliced form of XBP1, possibly at both the mRNA and protein levels, plays an important role in the inhibition of ERSE-dependent transcription during glucose deprivation.

We also tested whether ectopic expression of either the full-length or active (shorter) form of ATF6 had any effect on the inhibitory activity of VST by using pGRP78pro160-Luc reporter assays (Fig. 4, c–f). Co-transfection of HT1080 cells with 300 ng of pATF6(F), which encodes the full-length form of ATF6, enhanced basal reporter gene activity (Fig. 4, c). Reporter gene activity was further increased by 2DG treatment, and this 2DG-induced activity was less sensitive to VST than that in mock-transfected cells (Fig. 4, c). The induction of VST resistance was dependent on the amount of transfected ATF6(F) plasmid and required at least 10 ng (Fig. 4, d), an amount that led to much higher expression of full-length ATF6 protein than the endogenous level seen in mock-transfected cells (Fig. 4, e). Co-transfection of HT1080 cells with pATF6(A), which encodes the active form of ATF6, also led to VST resistance in 2DG-treated cells (Fig. 4, f), suggesting that proteolytic activation of the full-length form of ATF6 mediates the induction of resistance in 2DG-treated cells overexpressing full-length ATF6. These results suggest that a relatively low level of expression of endog-
enous ATF6 is necessary for VST to effectively inhibit ERSE-dependent transcriptional activation in glucose-deprived cells.

**Glucose-Regulated UPR-Inhibiting Activity**

We next examined whether the UPR-inhibiting activity of VST was seen specifically in glucose-deprived cells. Immunoblot analysis of lysates from both HT1080 and HT-29 cells demonstrated that VST suppressed induction of GRP78 and GRP94 protein accumulation even in the presence of TM, as long as the cells were deprived of glucose (Fig. 5, a, left, and data not shown). GRP expression was inhibited at essentially the same VST concentrations in the presence or absence of TM, suggesting that the inhibitory activity of VST was strictly dependent on glucose deprivation. Indeed, the inhibition of TM-dependent GRP induction by VST was eliminated as the amount of glucose increased (Fig. 5, a, right).

We also found that, during glucose deprivation, VST inhibited induction of ATF4 protein (Fig. 5, a, left), a downstream transcription factor in the PERK signaling pathway (29). VST inhibition of ATF4 expression also depended strictly on glucose deprivation. Interestingly, in the presence of glucose, VST induced ATF4 protein accumulation. Because ATF4 induction occurs under conditions of diverse (and seemingly unrelated) forms of stress (30), this observation raises the possibility that VST can stimulate other stress-signaling pathway(s) and that its mode of action changes depending on cellular glucose availability.

**Selective Killing of Glucose-Deprived Cells**

We next examined the effects of VST on cell viability. Under normal growth conditions, 24 hours of VST treatment of HT-29 cells had only a weak effect on cell viability, with approximately 30% of VST required to inhibit colony formation by 50% (IC50). By contrast, VST was highly toxic in cells exposed to glucose-free or 2DG-containing medium, resulting in approximately 30-fold lower IC50 (~1 μM) (Fig. 5, b, left and middle). Under hypoglycemic conditions, the cytotoxic activity of VST was associated with the inhibition of GRP expression (Fig. 1). By contrast, there was no consistent combined effect of VST with the chemical stressor TM (Fig. 5, b, right). HT1080 cells were similarly sensitized to VST under glucose starvation conditions, as indicated by flow cytometric assays of apoptotic cells show-
ing sub-G1 DNA content (Fig. 5, c) and of dead cells stained with 7-amino-actinomycin D (Fig. 5, d and e). VST-induced cell death was not substantial within 24 hours (Fig. 5, c), however, indicating that inhibition of GRP expression preceded cell death. Furthermore, induction of cell death by VST was seen at glucose concentrations of 0.1 mg/mL or lower (Fig. 5, d), concentrations at which GRPs were induced in a VST-suppressible manner (Fig. 5, a, right). We also found that VST-induced cell death under glucose starvation was not influenced by the further addition of TM (Fig. 5, e). Collectively, therefore, these data indicate that VST selectively kills glucose-deprived cells by disrupting the UPR.

We evaluated antiproliferative activity of VST with a panel of 39 human cancer cell lines (23–25). Across the entire cell line panel, the average VST concentration at which growth was inhibited by 50% (GL50) was 2.3 μM (for a 48-hour exposure). The COMPARE analysis revealed that the differential growth inhibition pattern of VST was unique, with little similarity to the patterns of more than 400 standard agents, which also suggests that VST has a unique mode of action. In the cell line panel analysis, VST had antiproliferative effects on most of the cell lines in the presence of glucose; the stomach cancer cell line MKN74 was one of the most sensitive lines, with a GL50 of 0.42 μM. MKN74 cells treated with VST for 48 hours showed little apoptosis under normal growth conditions; by contrast, when glucose was withheld for 48 hours, extensive apoptosis occurred (Fig. 6, a). Similarly, in the colony formation assay, a 24-hour treatment of VST was highly cytotoxic to glucose-deprived
MKN74 cells (Fig. 6, b). Therefore, the strong cytotoxic effect of VST on MKN74 cells was evident only during glucose deprivation, although VST had some antiproliferative effects in the presence of glucose. At doses that resulted in strong cytotoxicity during glucose deprivation, VST also inhibited GRP78 induction in MKN74 cells (Fig. 6, c).

Compared with HT1080 and HT-29 cells, MKN74 cells were the most sensitive to VST cytotoxicity during glucose deprivation (compare Figs. 6, b and 5, b). We therefore tested the in vivo antitumor activity of VST on MKN74 xenograft tumors. Once tumor volumes reached approximately 100 mm³, animals were treated intravenously (in groups of six) with VST. VST (at 13.5 or 18 mg/kg) inhibited tumor growth, with statistically significant differences in tumor volume between control (saline)- and VST-treated groups on day 11 (Fig. 6, d). To further explore the anticancer utility of VST in vivo, we administered the drug (13.5 mg/kg) in combination with cisplatin (7 mg/kg). The combination had better antitumor activity than that of control treatment and led to statistically significantly smaller tumor volumes over the long term (from days 11 to 24) than control treatment (Fig. 6, e). For example, the mean tumor volumes of the control and combination treatments were 387.6 mm³ (95% confidence interval [CI] = 311.6 to 463.6) and 205.1 mm³ (95% CI = 175.1 to 235.1), respectively, at day 11 (P < .001) and 819.8 mm³ (95% CI = 610.8 to 1028.8) and 389.4 mm³ (95% CI = 300.5 to 478.3), respectively, at day 24 (P = .004). Single-agent treatment with cisplatin also reduced tumor volumes, and statistically significant differences (versus control) were observed up to day 11 (P = .022 at day 11) (Fig. 6, e). Under these experimental conditions, tumor volume reduction was similar between mice treated with VST or cisplatin alone (Fig. 6, e). There were no deaths due to toxicity among mice receiving either single-agent or the combination treatment. The weights of mice treated with cisplatin alone or the combination decreased slightly by day 8, but they recovered afterward (Fig. 6, e, lower panel). These results suggest that VST had a therapeutic effect at well-tolerated doses.

**DISCUSSION**

We have presented evidence that VST shows highly selective cytotoxicity to glucose-deprived tumor cells that is associated with inhibition of the UPR. Specifically, VST inhibited expres-
sion of the UPR target genes GRP78 and GRP94 and repressed the production of the UPR transcriptional activators XBP1 and ATF4 during glucose deprivation. Moreover, VST showed in vivo antitumor activity at well-tolerated doses.

In mammalian cells, the regulation of the UPR is a complicated process that involves three independent signaling pathways: ATF6, IRE1, and PERK. Indeed, cells defective for ATF6 cleavage show no induction of GRP78 mRNA but can still induce XBP1 mRNA expression (10,15). Cells deficient in IRE1α or both IRE1α and IRE1β display normal GRP78 induction and survive under conditions of ER stress (15,31,32). PERK−/− cells show reduced survival, with defects in expression of various UPR target genes, but they do not show attenuated protein synthesis during ER stress (17,28). This complexity has been an obstacle to developing UPR-targeted drugs.

Nevertheless, it is likely that the production and/or quality of UPR transcriptional activators can be governed by a single signaling pathway, as seen in cells that lack functional GADD34, a downstream target gene product of the PERK signaling pathway (33). Indeed, GADD34-mutated cells show loss of GRP78 gene expression and impaired induction of the UPR transcriptional activators XBP1 and ATF4 (33). Furthermore, GADD34-mutated cells display persistent repression of protein synthesis and reduced cell survival in response to the chemical stressor thapsigargin (33), like cells treated with VST during glucose deprivation. The findings in GADD34 mutant cells indicate that disruption of the UPR, which leads to reduced cell survival during ER stress, can be achieved by mutation of a single gene and likely by a single compound. Therefore, the consistent observations between GADD34-mutated and VST-treated cells support a link between the cytotoxic and the UPR-inhibiting activities of VST during glucose deprivation. At present, however, we cannot rule out the possibility that mechanism(s) other than prevention of the UPR account for the biologic and therapeutic activity of VST. It will be important to clarify the precise mechanisms by which VST affects the UPR.

In addition to showing that the UPR is a potential target for cancer treatment, our findings have another important implication, namely that they provide insights into how to reduce resistance to chemotherapy. It is widely recognized that hypoglycemia, as well as hypoxia, induces resistance to chemotherapy, the principal problem in treating most common solid tumors (2,3,4,35). Indeed, under hypoglycemic conditions, a variety of human cancer cells are resistant to many clinically important antitumor drugs, as described previously (21,36). The induction of resistance is associated with the induction of the UPR target genes GRP78 and GRP94 (37,38). GRP78 and GRP94 show an anti-apoptotic function (7,39) and are induced in malignant cells (7,40,41). Therefore, selective inhibitors of the ER stress response, such as VST, may be useful to eliminate otherwise drug-resistant, hypoglycemic tumor cells. In addition, the severe hypoglycemic conditions under which VST becomes toxic are not observed in normal tissue. Thus, VST may be an attractive tool for exploring the potential of hypoglycemia-targeted therapy against solid tumors.

REFERENCES


NOTES

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Manuscript received November 27, 2003; revised June 28, 2004; accepted June 29, 2004.