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Blockade of Survival Response to Glucose Deprivation for Selective Killing of Tumor Cells

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GRP78 acts as a molecular chaperone in endoplasmic reticulum (ER) by associating transiently with incipient proteins as they traverse the ER and aiding in their folding and transport[1-3]. Furthermore, the GRP78 protein is also induced under various stress condition such as glucose starvation, inhibition of protein glycosylation by tunicamycin, perturbation of ER function and protein movement by brefeldin A, and suppression of ER-calcium-ATPase pump by thapsigargin[4, 5]. The enhancement of ER stress response (also known as the unfolded protein response) takes part in the resistant mechanism against chemotherapy and hypoxic stress in solid tumor[6]. While, the reduction of ER stress response involves in the pathology of central nervous diseases such as Alzheimer's disease and Parkinson's disease[7]. The ER stress response causes an increase in gene expression of a number of ER chaperones such as GRP78/Bip and GRP94[1]. Thus, substances that directly down- and up- regulate *grp*78 transcription are expected to be useful drugs for the treatment of cancer and Alzheimer's disease, respectively.

In the course of our screening program for chaperone modulators, we employed the reporter gene assay system utilizing luciferase gene. HeLa cells, which are transformed with luciferase gene under the control of *grp*78 promoter designated as HeLa 78C6 cells[2], respond sensitively to luciferase *grp*78 induction by ER stress such as the treatment of tunicamycin. By using this screening system, we isolated a several compounds such as pyrisulfoxin[8] and alternariol[9]. Further screening resulted in the isolation of a novel compound designated as versipelostatin (VST) from *Streptomyces versipellis* 4083-SVS6 as a down-regulator of the *grp*78 gene (Fig. 1). We report herein the fermentation, isolation and structure determination and brief biological activity o VST.

The versipelostatin producing strain 4083-SVS6, identified as *Streptomyces versipellis*, was cultivated in a seed medium consisting of starch 1.0%, polypepton 1.0%, molasses 1.0%, meat extract1.0% (pH 7.2) for 3 days at 27°C on a rotary shaker. The seed culture was inoculated into a production medium composed of starch 2.5%, soybean meal 1.5%, dry yeast 0.2%, $CaCO_3$ 0.4% (pH 7.0) and cultivated on a rotary shaker (200 rpm) for 5 days at 27°C. The active principle was extracted from the supernatant of cultural broth with EtOAc. The solvent layer was dried over Na_2SO_4, and concentrated to give an oily residue. The residue was subjected to a silica gel column chromatography using $CHCl_3$ - MeOH (20 : 1) as a solvent system. The

active fraction was concentrated under reduced pressure and purified by preparative HPLC using a PEGASIL ODS column (Senshu-Pak, 20 i. d. x 250 mm) developed with 80% MeOH.

The molecular formula of VST was established as $C_{61}H_{94}O_{17}$ by high-resolution FAB-MS spectrum [$(M+Na)^+$, *m/z* 1121.6398 (+ 0.9 mmu error)]. IR absorptions at 3400 and 1760 cm^{-1} implied the presence of hydroxyl groups and γ-lactone, respectively. The characteristic UV absorption maxima in MeOH at 250 and 270 nm indicated the presence of an α-acyltetronic acid chromophore, which was typical value for α -acyltetronic acids involved in kijanimicin and tetrocarcins[10, 11].

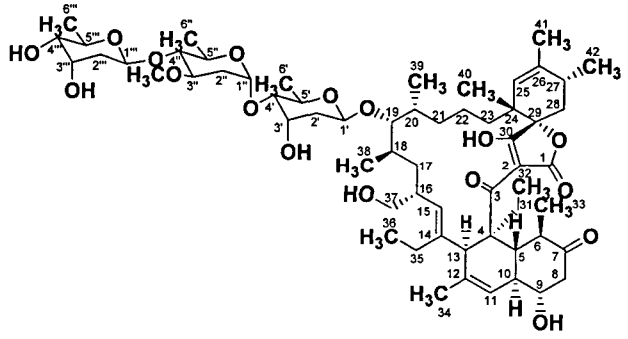

Fig. 1 Absolute structure of versipelostatin.

To determine whether VST affects endogenous GRP78 gene expression, we carried out a semi-quantitative RT-PCR analysis of RNA isolated from human colon carcinoma HT-29 and fibrosarcoma HT1080 cells. We found that VST suppressed GRP78 mRNA induction in both glucose-starved HT-29 and HT1080 cells (Fig. 2a,b), but did not affect the expression levels under normal growth conditions (Fig. 2b, control lanes). Likewise, VST inhibited GRP94 mRNA expression induced by glucose starvation (Fig. 2a,b). Immunoblot analysis of lysates from the glucose-starved cells showed that VST also suppressed protein accumulation of GRP78 and GRP94 (Fig. 2d).

To investigate whether the effect of VST on GRP expression extended to divergent ER stress stimuli, we used three different types of chemical stressors-hypoglycemia-mimicking agent 2-deoxyglucose (2DG), *N*-glycosylation inhibitor tunicamysin (TM), and calcium ionophore A23187. As expected, VST suppressed 2DG-induced GRP mRNA (Fig. 2b) and protein expressions (Fig. 2d) at the same concentrations for glucose starvation. The time-course experiments revealed that the inhibitory effect was seen from the onset of GRP mRNA induction and was maintained over the time periods examined (Fig. 2c, *left*). Unexpectedly, when cells were stressed by TM or A23187, VST had no effect on GRP mRNA expressions at any of the concentrations (Fig. 2b) and time points examined (Fig. 2c, *right*), nor on the protein accumulation (Fig. 2d).

To further define the specificity of the inhibitory effects of VST, we examined HSP70 mRNA expression, which represents another class of stress-inducible molecular chaperons. The changes in HSP70 mRNA expression varied under different stress conditions: glucose deprivation increased, but 2DG and TM decreased HSP70 expression levels (Fig. 2a,b). VST had no effect on the changes in HSP70 expression in the glucose-deprived and the TM-stressed cells. In the 2DG-stressed cells, a higher concentration of VST (10 μM)

strongly induced HSP70 gene expression. Thus, VST was incapable of inhibiting the HSP70 gene expression. Taken together, these results indicate that VST selectively inhibits ERSE-dependent transcription during glucose deprivation.

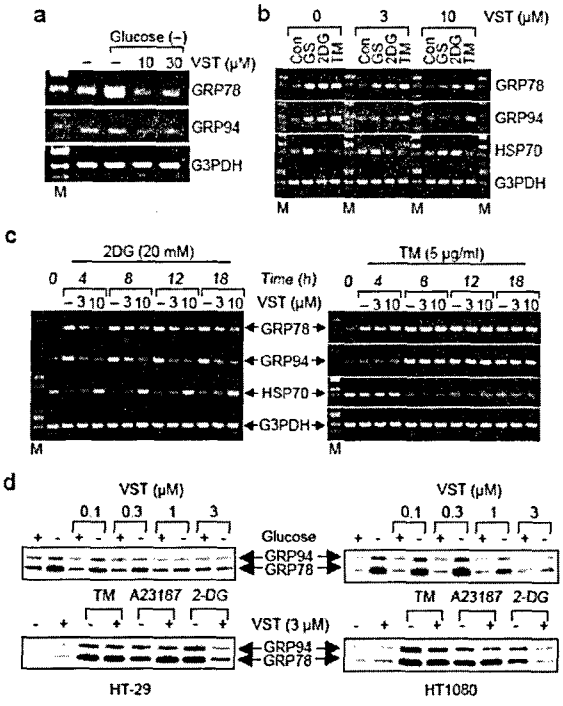

Fig. 2 VST prevents induction of GRP78 and GRP94.
a-d, RT-PCR (a-c) and immunoblot (d) analyses of GRP78, GRP94 and HSP70 expression levels. HT-29 (a,d) and HT1080 cells (b-d) were treated for 18 h or the indicated time periods with VST under normal (Con) or stress conditions, as indicated. GS, glucose starvation; M, marker.

We next examined whether the UPR-inhibiting activity of VST was associated with glucose availability for cells. Immunoblot analysis of lysates from both HT1080 and HT-29 cells demonstrated that VST suppressed induction of GPR78 and GRP94 even in the presence of TM as long as glucose was withheld (Fig. 3a, *left* and not shown). GRP expression was inhibited at essentially the same concentrations in the presence or absence of TM, suggesting that the inhibitory activity of VST strictly depended on glucose deprivation. Indeed, the inhibition of TM-dependent GRP induction by VST was eliminated as the amounts of glucose increased (Fig. 3a, *right*).

We also found that during glucose deprivation the VST-treated cells lacked ATF4 protein induction (Fig 3a, *left*), a downstream transcription factor in the PERK-signaling pathway. This VST activity also strictly depended on glucose deprivation. Interestingly, VST showed an ATF4-inducing activity in the presence of glucose. Because ATF4 induction occurs under conditions of diverse and seemingly unrelated forms of stress, this observation raises the possibility that VST could stimulate other stress-signaling pathway(s) and that its mode of action changes depending on cellular glucose availability.

We next examined the effects of VST on cell viability. Under normal growth conditions, a 24-h, VST treatment of HT-29 cells had only a weak effect on cell viability; ~30 μM of VST was required to inhibit

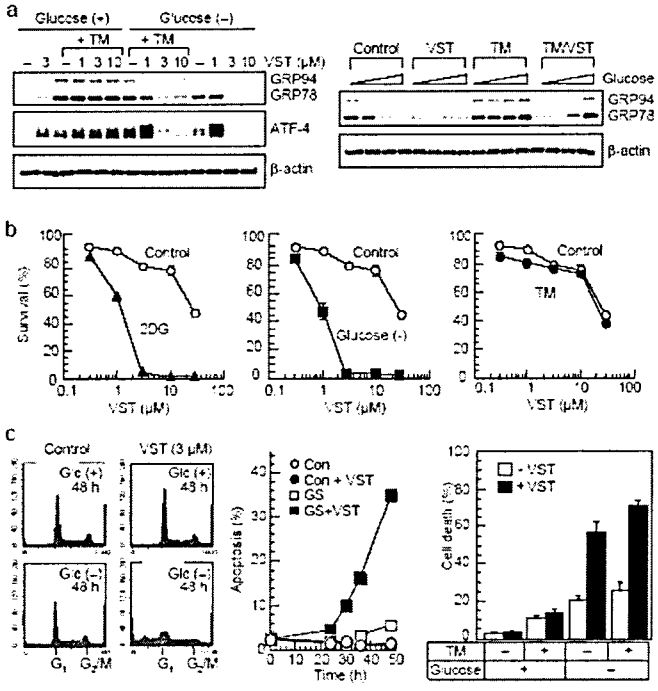

Fig. 3 Glucose-dependent action of VST.

a, Immunoblotting of GRP78, GRP94 and ATF4 for HT1080 cells at 18 h after VST (*right*, 3 μM) *plus* TM or no stressor were added to normal (2 mg glucose/ml), glucose-free medium (*left*) or to medium containing graded concentrations of glucose (0, 0.1, 0.5, 2 mg/ml; *right*). **b**, Colony formation analysis of HT-29 cells after a 24-h VST treatment under normal (control) or stress conditions as indicated. **c**, Flow-cytometric analysis of apoptotic (*left* and *middle*) and dead populations (*right*) in HT1080 cells at 48 h (*left* and *right*) or the indicated time points (*middle*) after the addition of VST (3 μM) with or without glucose (GS). Dead cells were also determined with or without TM (*right*).

colony formation by 50% (IC_{50}). The VST treatment became highly toxic in the glucose-free or 2DG-containing medium, resulting in approximately 30-fold lower IC_{50} (~1 μM) under hypoglycemic conditions (Fig. 3b, *left* and *middle*). Under the same conditions, cytotoxic activity correlated well with the inhibition of GRP expression (see Fig. 1) and there was no consistent, combined effect of VST with the chemical stressor TM (Fig. 3b, *right*). The similar sensitization of HT1080 cells to VST was observed under glucose starvation, as determined by flow cytometric assays of either apoptotic cells showing sub-G1 DNA content (Fig. 3c, *left* and *middle*) or dead cells stained with 7-amino-actinomycin D (Fig. 3c, *right*). The VST-induced cell death was not significant within 24 h (Fig. 3c, *middle*), indicating that GRP expression inhibition preceded the cell death. We also found that VST-induced cell death under glucose starvation was not influenced by further addition of TM (Fig. 3c, *right*). The present data collectively indicate that VST selectively kills glucose-deprived cells by disrupting the UPR.

References

1. Kaufman, R. J. *Genes Dev.* 1999, 1211-1233.
2. Yoshida, H.; Haze, K.; Yanagi, H.; Yura , T.; Mori, K. *J. Biol. Chem.* 1998, *273*, 33741-33749.

3. Roy, B.; Lee, A. S. *Nucleic Acids Res.* 1999, *27*, 1437-1443.
4. Wang, Y.; Shen, J.; Arenzana, N.; Tirasophon, W.; Kaufman, R. J.; Prywes, R. *J. Biol. Chem.* 2000, *275*, 27013-27020.
5. Clapham, D. E. *Cell* 1995, *80*, 259-268.
6. Katschinski, D. M.; Jacobson, E. L.; Wiedmann, G. J.; Robins, H. I. *J. Cancer Res. Clin. Oncol.* 2001, *127*, 425-435.
7. Imaizumi, K.; Miyoshi, K.; Katayama, T.; Yoneda, T.; Taniguchi, M.; Tohyama, M. *Biochimica et Biophysica Acta* 2001, *1536*, 85-96.
8. Tsuge, N.; Furihata, K.; Shin-Ya, K.; Hayakawa, Y.; Seto, H. *J. Antibiot.* 1999, 52, 505-507.
9. Sjoland, S.; Gatenbeck, S. *Acta Chem. Scand.* 1966, 20, 1053-1059.
10. Mallams, A. K.; Puar, M. S.; Rossman, R. R.; McPhail, A. T.; Macfarlane, R. D. *J. Am. Chem. Soc.* 1981, *103*, 3940-3943.
11. Tamaoki, T.; Kasai, M.; Shirahata, K.; Ohkubo, S.; Morimoto, M.; Mineura, K.; Ishii, S.; Tomita, F. *J. Antibiot.* 1980, 33, 946-950.