Inhibition of Cancer Cell Growth by GRP78 siRNA Lipoplex via Activation of Unfolded Protein Response

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Proteasome inhibitors are a novel class of molecular-targeted anti-cancer drugs that suppress the degradation of malfolded proteins, trigger endoplasmic reticulum (ER) stress, and activate apoptosis signals. Glucose-regulated protein 78 (GRP78), a major ER chaperone, is one of the most important molecules for transduction of unfolded protein response (UPR) signals. In accordance with past findings that expression of GRP78 is elevated in cancer cells and helps to resist stress-induced apoptosis, GRP78 knockdown could be effective in anticancer therapy. We tested this hypothesis and found that transfection of small interfering RNA (siRNA) targeting GRP78 inhibited the growth of RENCA renal carcinoma cells, in association with elevated gene expression of UPR downstream signaling molecules (CHOP, EDEM1, and ERdj4 mRNA). In addition, the combinatorial effect of GRP78 siRNA with ER stress inducers (tunicamycin, MG132, and 2-deoxyglucose) on survival was measured. Combination of GRP78 siRNA and the ER stress inducers more extensively reduced cell viability than combination with scrambled siRNA. Besides RENCA, B16BL6 melanoma cells were also shown to be sensitive to GRP78 siRNA. These results suggest that GRP78 knockdown might be an effective strategy for cancer therapy targeting UPR-induced apoptosis.

Key words glucose-regulated protein 78; small interfering RNA; apoptosis; unfolded protein response; endoplasmic reticulum stress; cancer cell

The endoplasmic reticulum (ER) is responsible for protein synthesis, conformational maturation, and quality control of correctly folded proteins. In cancer cells, protein synthesis and by-product disposal are elevated, therefore, disruption of ER functions is believed to be a promising approach for cancer therapy. Proteasome inhibitors, which are a novel class of molecular-targeted anti-cancer drugs, suppress the degradation of malfolded proteins and trigger ER stress, which leads to the activation of the unfolded protein response (UPR) and subsequent apoptotic signals. 1, 2 Bortezomib was the first approved proteasome inhibitor, and is used for the treatment of relapsed multiple myeloma 3 and mantle cell lymphoma. 4 A second generation of proteasome inhibitors are now under development to improve drug efficacy and compliance. 5

Molecular chaperones are key factors involved in unfolded protein accumulation. Glucose-regulated protein 78 (GRP78) is known as a stress-inducible ER chaperone protein and serves as an ER stress signal regulator. 5 Under unstressed conditions, GRP78 associates with ER transmembrane sensor proteins, such as protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme-1 (IRE1) and activating transcription factor 6 (ATF6), 6, 7 and these interactions maintain them in an inactive form. However, accumulation of unfolded proteins results in the detachment of GRP78 from the sensor proteins and the subsequent activation of the unfolded protein response (UPR), which results in general translational attenuation, up-regulation of chaperones and folding enzymes, and enhanced ER-associated degradation (ERAD) of malfolded proteins. 9 The UPR is fundamentally a cytoprotective response, however, its excessive or prolonged signaling leads to cell death through the activation of apoptotic pathways. 10

Inhibition or down-regulation of GRP78 has been demonstrated to increase ER stress-induced cell death in melanoma and cancer cells. 11, 12 However, the induction of apoptosis by GRP78 small interfering RNA (siRNA) transfection is not observed in some cell types, especially those with low GRP78 expression. 13 The present study was carried out to confirm the feasibility of GRP78 targeting for the inhibition of cell growth. We used the renal carcinoma cell line RENCA, which highly expresses GRP78 basally, to investigate the effects of GRP78-targeted siRNA (hereafter called “GRP78 siRNA”) on their proliferation rate and gene expression profile of UPR signaling pathway molecules. Moreover, to investigate intercell differences, the inhibitory effect of GRP78 siRNA on cell viability of three additional cell lines with varying basal levels of GRP78 was examined. Our findings suggest that the effectiveness of GRP78 siRNA likely depends on the activation of UPR signaling rather than the basal level of GRP78. We also demonstrate that GRP78 siRNA enhances the efficacy of ER stress inducer-mediated inhibition of cell survival.

MATERIALS AND METHODS

Cell Culture B16BL6 mouse melanoma, Colon26 mouse adenocarcinoma, and RENCA mouse renal carcinoma cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, U.S.A.). PAN02 pancreatic ductal adenocarcinoma cells were obtained from the National Cancer Institute (Frederick, MD, U.S.A.). B16BL6 and RENCA cells were routinely grown in Dulbecco’s modified Eagle’s medium

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After incubation, the WST-1 cell viability test was carried out of cell culture medium and incubated for an additional 24 h. Nicamycin, 2-deoxyglucose, or MG132 was added to 100 desired concentrations. Cells were cultured in 96-well plates phosphate-buffered saline (Nissui Pharmaceutical) to the (Wako Pure Chemical Industries, Ltd.) were dissolved in 2-deoxyglucose (Nacalai Tesque, Kyoto, Japan), and MG132 was added to 100 µM and incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

In Vitro Gene Transfection GRP78 siRNA (sense 5'-gaaugaauagagcauaut-3' and antisense 5'-aauuccuauaauauucct-3') and corresponding scrambled siRNA (sense 5'-aauuccuauaauauauucct-3' and antisense 5'-acccauaggacauauuucct-3') were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Reverse transfection was carried out following the vendor’s protocol for RNAiMAX (Invitrogen, Carlsbad, CA, U.S.A.). Briefly, the stock solution of siRNA duplex was diluted in Opti-MEM (Invitrogen) medium to yield a concentration of 10 nm and the mixture was then aliquoted in multiwell cell culture plates. The lipofectamine reagent RNAiMAX was then added to each well containing the siRNA solution at a ratio of 1:100 (v/v), and incubated for 20 min at room temperature. Then, an appropriate number of cells suspended in Opti-MEM were added to the cell culture plates. Twenty-four hours later, Opti-MEM was replaced with the appropriate regular fresh culture medium as mentioned above.

Cell viability tests were carried out using GRP78 siRNA, and the scrambled siRNA, was counted each day up to 4 d post-transfection according to the Trypan Blue staining method. Briefly, 1×10⁶ RNAiMAX cells were plated and reverse transfected with 10 nm GRP78 siRNA and 5 µL RNAiMAX in 3 mL of Opti-MEM in 6-well culture plates. For counts, cells were trypsinized with 0.4% Difco™ Trypsin 250 (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) supplemented with 0.02% ethylenediaminetetraacetic acid (EDTA), and subjected to cell counting using a hemocytometer.

WST-1 Assay Cell viability tests were carried out using WST-1 cell proliferation reagent (Roche Diagnostics, Indianapolis, IN, U.S.A.). Cells (8×10⁵ cells/well) were plated in 96-well culture plates, transfected with 10 nm GRP78 siRNA and 0.2 µL RNAiMAX in 120 µL Opti-MEM, and then cultured for either 2 or 4 d in serum-containing culture media. After the relevant time point, the culture medium was replaced with 100 µL fresh culture medium supplemented with 10 µL WST-1 assay reagent and incubated for 20 min. The plates were then read on an Eon Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, U.S.A.) at both 450 nm and 620 nm for measurement of the absorbance associated with WST-1 reagent and background, respectively. Cell viability was compared between GRP78 siRNA and scrambled siRNA transfection groups.

Cell Culture with ER Stress Inducers Tunicamycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2-deoxyglucose (Nacalai Tesque, Kyoto, Japan), and MGI32 (Wako Pure Chemical Industries, Ltd.) were dissolved in phosphate-buffered saline (Nissui Pharmaceutical) to the desired concentrations. Cells were cultured in 96-well plates for 3 d after transfection with GRP78 siRNA, then 20 µL tunicamycin, 2-deoxyglucose, or MGI32 was added to 100 µL of cell culture medium and incubated for an additional 24 h. After incubation, the WST-1 cell viability test was carried out as described above.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Cells were plated in 6-well culture plates (1×10⁵ cells/well), and transfected with 10 nm siRNA and 5 µL of RNAiMAX in 3 mL of Opti-MEM. Twenty-four hours later, total RNA was isolated from cells using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, U.S.A.). Reverse transcription of mRNA was carried out using 1 mg isolated cDNAs and PrimeScript RT reagent Kit (TaKaRa Bio, Shiga, Japan). The detection of GRP78, CHOP, EDEM1, ERdj4, and glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was carried out by real-time PCR using SYBR Premix Ex Taq (TaKaRa Bio) and the Lightcycler Quick System 350S (Roche Diagnostics, Indianapolis, IN, U.S.A.). The primers for GRP78, CHOP, EDEM1, ERdj4 and GAPDH were constructed as follows: GRP78, 5'-ctcgagccttgtggaagagg-3' (forward) and 5'-ctatgattctaggcagcag-3' (reverse); CHOP, 5'-gaagagagagagaaagcgaag-3' (forward) and 5'-ggagagacagagagggtcag-3' (reverse); EDEM1, 5'-agcaaccaacagtcctctcag-3' (forward) and 5'-ctcgagctctaaatcaactc-3' (reverse); ERdj4, 5'-acagaagcggagtatgctt-3' (forward) and 5'-tgctgttgttgtttatcaacag-3' (reverse); and GAPDH, 5'-ctcagctgcaacactc-3' (forward) and 5'-ggagggctcagcag-3' (reverse) (Invitrogen).

Western Blotting Analysis Cells cultured in 6-well cell culture plates were harvested on day 2 post-transfection and homogenized with a lysis buffer consisting of 250 mM sucrose, 10 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), 1 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N’,N’’-tetraacetic acid (EGTA), and Protease Inhibitor Cocktail (Sigma-Aldrich), followed by centrifugation at 15000×g for 10 min. The samples were diluted in a 100 mM Tris–HCl pH 6.8 buffer supplemented with 12% 2-mercaptoethanol, 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 0.004% bromophenol blue, and boiled at 95°C for 5 min. Proteins were resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE) (SuperSep™ Ace 10%; Wako Pure Chemical Industries, Ltd.) and transferred onto polyvinylidene difluoride (PVDF) membranes (Hybond-P; Amersham Biosciences, Buckinghamshire, U.K.). The membranes were then blocked with 3% bovine serum albumin (BSA) in 25 mM Tris-buffered saline containing 0.1% Tween (TWEEN-TBS) overnight. Next, membranes were incubated for 1 h with an anti-GRP78 or anti-β-actin antibody (Abcam, Cambridge, U.K.) in Tween-TBS containing 1% BSA, followed by four washes with BSA-free Tween-TBS, and incubation for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Abcam) in Tween-TBS containing 1% BSA. Protein bands were visualized by Luminate™ Forte Western HRP Substrate (Millipore Corporation, Billerica, MA, U.S.A.) and the luminescence was detected by the ImageQuant LAS4000 (GE Healthcare, Uppsala, Sweden).
RESULTS

Effect of GRP78 siRNA on the Proliferation and Survival of RENCA Cells

To test the effect of GRP78 siRNA on RENCA cell proliferation and survival, the number of RENCA cells was counted daily, up to 4 d, following transfection with GRP78 siRNA (Fig. 1A). The doubling time of RENCA cells was approximately 1 d in the absence of treatment. From day 3 onwards, a statistically significant decrease was observed between the number of cells that received GRP78 siRNA and those that were transfected with scrambled siRNA. Of note, the rate of growth of control transfected cells tended to be lower than that of non-treated cells, presumably owing to the mildly toxic effect of lipofection. To examine whether GRP78 siRNA reduced cell survival, the WST-1-based cell viability assays were performed on days 2 and 4 after transfection (Fig. 1B). On both days, especially day 4, the viability of RENCA cells was significantly inhibited by GRP78 siRNA transfection compared with that of the scrambled siRNA control. Knockdown of GRP78 by the targeted siRNA was observed on day 2 following transfection as measured by Western blot analysis (Fig. 1C).

Effect of GRP78 siRNA on the Expression of UPR-Related Genes in RENCA Cells

The effect of GRP78 siRNA on the gene expression of representative UPR downstream signaling molecules, CHOP, EDEM1 (ER degradation-enhancing alpha-mannnosidase-like protein), and ERdj4 (mammalian DnaJ/HSP40-like protein) was investigated using quantitative RT-PCR. CHOP, also known as GADD153 or DDIT3, encodes a member of the CCAAT/enhancer-binding protein family and regulates transcription of proteins associated with apoptosis signaling pathways. In contrast, EDEM1 and ERdj4 are known to be involved in cell survival by activation of ERAD. Fig. 1D shows the expression of these UPR-related genes in RENCA cells transfected with GRP78 (gray) and scrambled (white) siRNAs. The mRNA expression levels of these three proteins following GRP78 siRNA transfection were greater than those in the scrambled siRNA control. In particular, CHOP expression levels were approximately four times more than that of the control.

Effect of Combining GRP78 siRNA and ER Stress Inducers on Cell Survival

Tunicamycin, MG132, and 2-deoxyglucose are widely used as ER stress inducers, and function through inhibition of three processes: protein glycosylation, proteasome-mediated proteolysis, and glucose uptake, respectively. We examined whether GRP78 knockdown reduces ER stress resistance and thereby increases sensitivity to ER stress inducers in RENCA cells, by testing these inducers over a

![Figure 1](https://example.com/figure1.png)

(A) Proliferation of non-treated RENCA cells (△) or those transfected with either GRP78 siRNA (●) or scrambled siRNA (○) was measured at the indicated time points. Data represent mean with S.E.M. (n=3). (B) WST-1 assay was used to measure the viability of RENCA cells on days 2 and 4 post-transfection with GRP78 siRNA (gray) or scrambled siRNA (white). Data were normalized against GAPDH mRNA and expressed as a percentage of the scrambled siRNA. Data represent mean with S.E.M. (n=8). (C) Western blot analysis of GRP78 protein expression on day 2 post-transfection with the indicated siRNAs. β-actin served as a control for input. (D) Expression of CHOP, EDEM1, and ERdj4 mRNA was measured in RENCA cells by quantitative RT-PCR at 24 h post-transfection with GRP78 (gray) and scrambled (white) siRNAs. Data represent mean with S.E.M. (n=3) normalized to the scrambled siRNA data. Statistical comparisons were made using the Student’s t-test (*p<0.05 and **p<0.01, compared with scrambled siRNA).
range of concentrations for their effect on cell growth in the presence or absence of GRP78 siRNA. It was found that the growth of GRP78 siRNA transfected cells was more strongly suppressed compared with that of scrambled siRNA transfected cells when co-treated with any of the ER stress inducers (Fig. 2). Table 1 summarizes statistical analysis of combining GRP78 siRNA and ER stress inducers. To evaluate both primary and interactive effects, a two-way analysis of variance was conducted. As intuitively obvious from Fig. 2, the primary effects of GRP78 siRNA and ER stress inducers were statistically significant. In addition, a statistically significant interactive effect between the two was observed with tunicamycin. In case of MG132 and 2-deoxyglucose, their extensive primary effects might make it statistically difficult to detect interactive effects with GRP78 siRNA.

Effect of GRP78 siRNA on the Survival of Several Cancer Cell Lines In addition to RENCA cells, the effect of GRP78 siRNA transfection on survival was investigated in different cancer cell lines, B16BL6, Colon26, and PAN02. Figures 3A and 3B illustrates the viability of GRP78 siRNA-treated and scrambled siRNA-treated cells on days 2 and 4 post-transfection, respectively. B16BL6, as well as RENCA, were sensitive to GRP78 siRNA as suggested by the lower WST-1 staining, compared with the scrambled siRNA control. In Colon26 and PAN02 cells, however, there was no significant difference in survival between GRP78 siRNA-treated and scrambled siRNA-treated groups. The expression of CHOP mRNA following treatment with either GRP78 siRNA or scrambled siRNA was also examined (Fig. 3D). In B16BL6 cells, as in RENCA cells, the expression of CHOP mRNA was greatly increased by transfection with GRP78 siRNA compared with that of the scrambled siRNA control. These results suggest that among different cell lines, inhibition of cell viability by GRP78 siRNA correlates with an increase in CHOP mRNA expression.

Both basal and post-transfection levels of GRP78 mRNA expression were measured to evaluate the knockdown efficiency of GRP78 siRNA (Fig. 3C). RENCA cells showed the greatest basal expression of GRP78 mRNA, followed by Colon26, PAN02, and B16BL6 cells. In contrast, the most efficient knockdown of GRP78 siRNA was observed in the order B16BL6 (79.7%), RENCA (77.8%), Colon26 (32.0%), and finally PAN02 (20.1%) cells. These results indicate that the inhibitory effect of GRP78 siRNA on cell viability was correlated not with the basal GRP78 level but with the knockdown efficiency among different cell lines.

DISCUSSION

Cancer cells acquire stress resistance to survive in hostile microenvironments, such as those that are hypoxic and/or low in glucose. Recent studies indicate that GRP78 is one of the major players involved in protecting cancer cells from apoptosis, promoting metastasis, and allowing cancer cells to resist several anti-cancer drugs. Moreover, the expression of GRP78 has been shown to be elevated in a variety of tumors, including prostate, lung, breast, colon, and gastric tumors. According to these observations, GRP78 is a potential target for cancer therapy.

However, there have been controversial observations with respect to the targeting of GRP78 in cancer cells. For instance, while siRNA and inhibitors against GRP78 have been shown to elicit growth inhibition in melanoma and prostate cancer cells, they were ineffective in several other cancer cell lines despite decreased expression of GRP78. This is believed to be primarily due to the bilateral character of the UPR response, i.e., the activation of both survival and apoptotic pathways. To investigate the effect of GRP78 knockdown in detail, we used a renal carcinoma cell line, RENCA, that has been reported to show strong ER stress sensitivity to...
proteasome inhibitors. We found that the siRNA-mediated knockdown of GRP78 augmented mRNA expression levels of the ER stress-related genes, CHOP, EDEM1, and ERdj4 in RENCA cells (Fig. 1D). CHOP is a pro-apoptotic transcription factor that is induced mainly by PERK, while EDEM1 and ERdj4 are responsible for the induction of ERAD, expression of which is mediated through the XBP1 pathway. Thus, these data suggest that GRP78 siRNA activates both survival and apoptotic pathways in RENCA. Because cell growth was significantly inhibited by GRP78 siRNA (Fig. 1), it seems that the apoptosis signal, mediated by CHOP, might exceed that of ERAD in RENCA cells.

When four cell types varying in expression of GRP78 mRNA were tested, the effect of GRP78 siRNA on cell growth was different. As shown in Figs. 3A, B, only B16BL6 and RENCA cells had reduced cell viability as a result of GRP78 siRNA. Notably, this was in agreement with the degree of GRP78 knockdown (Fig. 3C). Indeed, the expression of GRP78 mRNA in B16BL6 and RENCA cells was more greatly decreased by GRP78 siRNA than in the other two cell lines. Together with up-regulation of CHOP mRNA (Fig. 3C), these data suggest that the apoptosis pathway is activated in these two cell types following transfection with GRP78 siRNA. The inhibitory effect of GRP78 siRNA on cell viability was also not necessarily associated with the basal level expression of GRP78. Instead, differences in cell viability in response to GRP78 siRNA could be caused by variability in transfection efficiency among the different cell types.

In a variety of cancer cell lines, solid tumors and human cancer biopsies, the level of GRP78 is highly elevated in correlation with drug resistance. In vitro studies have demonstrated that overexpression of GRP78 protects cells from chemotherapeutic agents, such as doxorubicin or cisplatin. Moreover, a retrospective cohort study revealed that in breast cancer patients who received doxorubicin-based chemotherapy, the GRP78-positive group showed an increased likelihood of disease recurrence. Therefore, it was assumed that concomitant administration of GRP78 siRNA with ER stress inducers may show a synergistic effect. We tested the effect of combining GRP78 siRNA with the ER stress inducers tunicamycin, 2-deoxy-D-glucose or MG132, which have been known to induce GRP78 expression. Indeed, combination of transfection with GRP78 siRNA and any of the ER stress inducers more extensively reduced cell viability than combination with scrambled siRNA (Fig. 2). These results suggest that over-expression of GRP78 function results in a cytoprotective effect against chemically induced ER stress, and that knockdown of GRP78 and subsequent UPR-induced apoptosis might be an effective strategy for cancer therapy.

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REFERENCES


