fect of GEM [5]. Our previous studies involved proteomic analysis of GEM-resistant and -sensitive human pancreatic adenocarcinoma cell lines. Two-dimensional gel electrophoresis showed upregulated or downregulated protein spots in GEM-resistant cell lines compared with GEM-sensitive cell lines, and they were identified by liquid chromatography-tandem mass spectrometry and Western blotting. Those results showed upregulation of heat shock protein (HSP) 27 in the GEM-resistant cell lines compared with the GEM-sensitive cell lines. The treatment of GEM-resistant cells with siRNA for HSP27 restored sensitivity to GEM. In addition, upregulated HSP27 in tumor specimens was associated with higher resistance to GEM and shorter survival in patients with pancreatic cancer [6, 7]. Further experiments showed that treatment with interferon-γ downregulated HSP27 and increased the cytotoxic effect of GEM on GEM-resistant KLM1-R cells [8]. KNK437 (N-formyl-3, 4-methylenedioxy-benzylidene-γ-butyrolactam) is a benzylidene lactam compound that was first synthesized by Kaneka Corporation [9]. It has been shown to inhibit the acquisition of thermotolerance in a dose-dependent manner by inhibiting several HSPs. The purpose of this study was to downregulate HSP27 expression in GEM-resistant KLM1-R cells by means of KNK437 treatment and to examine the effect of combined treatment with GEM and KNK437 on KLM1-R.

Materials and Methods

Tumor Cell Lines and Culture Conditions

Two human pancreatic cancer cell lines, GEM-sensitive KLM1 and GEM-resistant KLM1-R, were provided by the Department of Surgery and Science at the Kyushu University Graduate School of Medical Science. KLM1-R was established by exposing KLM1 cells to GEM, as described previously [10]. This GEM-resistant cell line did not show any morphological changes, including spindle-shaped morphology and appearance of pseudopodia such as epithelial-to-mesenchymal transition (data not shown). The GEM-resistant human pancreatic cancer cell line PK59 was kindly provided by the Institute of Development, Aging and Cancer at Tohoku University [7]. The cell lines were grown in RPMI-1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10% FBS. Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

Sample Preparation

Cells were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1% NP-40) on ice. Suspensions were incubated for 1 h at 4°C and centrifuged at 21,500 *g* for 30 min at 4°C, and the supernatants were stored at –80°C until use [11].

Western Blot Analysis

Fifteen micrograms of protein were used for Western blotting. After electrophoresis, gels were transferred electrophoretically onto PVDF membranes (Immobilon-P, Millipore, Bedford, Mass., USA) and blocked for 1 h at room temperature with TBS containing 5% skimmed milk. Primary antibodies were anti-HSP27 monoclonal antibody [dilution 1:200; sc-13132 (F-4) Santa Cruz Biotechnology, Santa Cruz, Calif., USA] and antiactin goat polyclonal antibody [dilution 1:200; sc-1616 (I-19) Santa Cruz Biotechnology]. Membranes were incubated with the primary antibody overnight at 4°C, washed 3 times with TBS containing 0.05% Tween-20 and once with TBS, then incubated with a horseradish peroxidase-conjugated secondary antibody [dilution 1:5,000; 115-035-003 (anti-mouse), No. 305-035-003 (anti-goat), Jackson Immunoresearch Laboratories Inc., West Grove, Pa., USA] for 1 h at room temperature and developed with a chemiluminescence reagent (ECL Western Blotting Detection Reagents, GE Healthcare) [12, 13].

Assay for the Effect of KNK437 on Proliferation of GEM-Resistant Cells

Proliferation of GEM-resistant KLM1-R cells was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega Corp., Madison, Wisc., USA) assay, which is based on the reduction of this tetrazolium salt by viable cells [14]. Cells (2×10^3 cells per well) were seeded in complete medium in 96-well plates, cultured for 24 h, then exposed to different concentrations (0, 10, 50, 100 μM) of KNK437 (Calbiochem, Tokyo, Japan) for 48 h and then treated with GEM (0 or 10 μg/ml; Eli Lilly and Company, Kobe, Japan) for 48 h. To determine the growth rate, triplicate wells were mixed with 20 μl of MTS solution. After 2 h, the optical density of the dissolved material was measured at 490 nm with a microtiter plate reader (Model 550 Microplate Reader, Bio-Rad, Hercules, Calif., USA). Results were derived from at least 3 independent sets of triplicate experiments. Statistical analysis of the differences in the percentage of control cell growth with or without GEM and KNK437 treatment was performed using one-way or two-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be statistically significant. The software application used was Excel Statistics 2008.

Results

Effect of the HSP Inhibitor KNK437 on HSP27 Levels in KLM1, KLM1-R and PK59 Cells

So far, some groups have reported that KNK437, an HSP inhibitor, inhibited hsp gene expression and function [15–18]. Therefore, we used KNK437 to increase the cytotoxic effect of GEM on GEM-resistant KLM1-R cells by downregulation of HSP27. Protein levels of HSP27 in KLM1-R, KLM1 and PK59 cells were examined by Western blotting. The intensity of the HSP27 band in untreated KLM1-R was 37.20. On the other hand, the intensities of the HSP27 bands in KLM1-R treated with 50 and 100

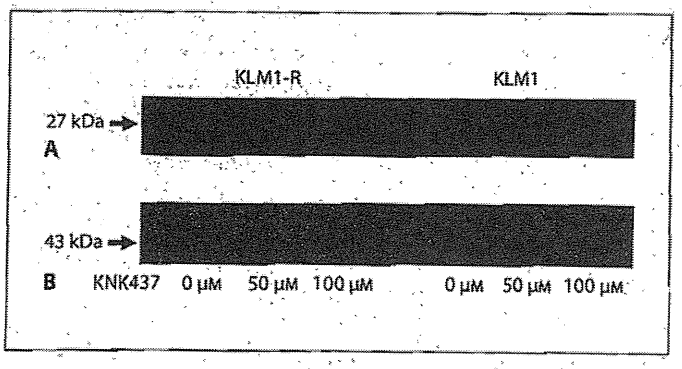

Fig. 1. Expression levels of HSP27 in KLM1 and KLM1-R exposed to KNK437. **A** The HSP27 expression level (27 kDa) was upregulated in KLM1-R compared with KLM1 and downregulated in the cells exposed to KNK437 (50 and 100 μM). **B** The actin expression level (43 kDa) was the same in all cells. Fifteen micrograms of protein were used.

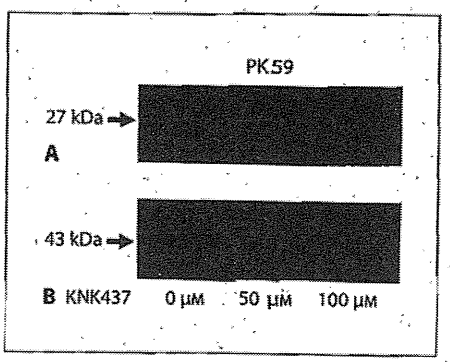

Fig. 2. Expression levels of HSP27 in PK59 exposed to KNK437. **A** The HSP27 expression level (27 kDa) in PK59 was downregulated in the cells exposed to KNK437 (50 and 100 μM). **B** The actin expression level (43 kDa) was the same in all cells. Fifteen micrograms of protein were used.

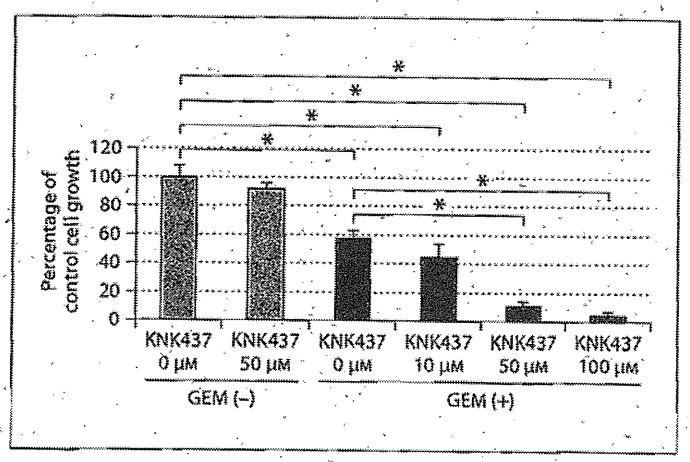

Fig. 3. The antitumor cytotoxic effect of KNK437 and GEM in combination on KLM1-R. This graph shows the antitumor cytotoxic effect of only GEM or a combination of GEM and KNK437 on KLM1-R cells. The growth of KLM1-R cells without treatment was taken as 100%. We found a strong antitumor cytotoxic effect on KLM1-R by combination therapy with GEM and KNK437. Statistical analysis of the differences in the percentage of control cell growth after treatment with or without GEM and KNK437 was performed using one-way ANOVA. A value of $p < 0.05$ was considered to be statistically significant. * $p < 0.01$.

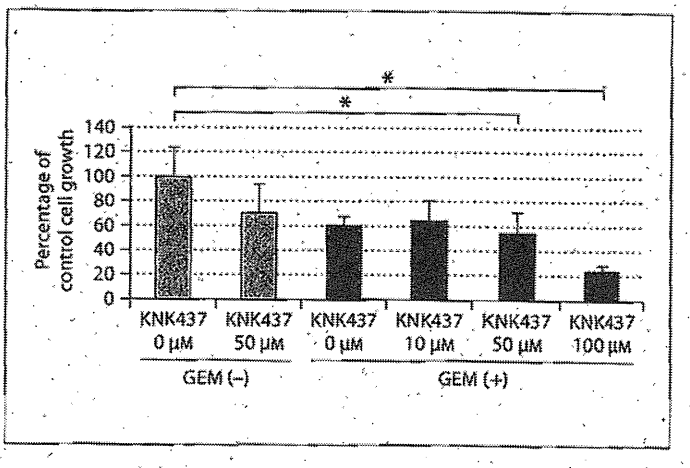

Fig. 4. The antitumor cytotoxic effect of KNK437 and GEM in combination on PK59. This graph shows the antitumor cytotoxic effect of GEM alone or in combination with KNK437 on PK59 cells. The growth of PK59 cells without treatment was taken as 100%. Statistical analysis of the differences in the percentage of control cell growth after treatment with or without GEM and KNK437 was performed using one-way ANOVA. A value of $p < 0.05$ was considered to be statistically significant. * $p < 0.01$.

μM KNK437 were 13.15 and 12.70, respectively. The intensity of the HSP27 band in untreated PK59 was 57.23. On the other hand, the intensities of the HSP27 bands in PK59 treated with 50 and 100 μM KNK437 were 23.10 and 15.33, respectively. The results showed that HSP27 protein level was dramatically reduced by KNK437 in KLM1-R, KLM1 and PK59 (fig. 1, 2).

The Antitumor Effect of KNK437 and GEM on KLM1-R and PK59

We examined the antitumor cytotoxic effect of combined treatment with GEM and KNK437 on GEM-resistant KLM1-R cells by the MTS assay. KLM1-R cells were exposed to KNK437 (0, 50, 100 μM) for 48 h and then treated with 10 μg/ml GEM for 48 h. Figure 3 shows the

antitumor cytotoxic effect of combined KNK437 and GEM treatment on GEM-resistant KLM1-R cells. The drug KNK437 did not affect the growth of the KLM1-R cells. The antitumor effect of the combined treatment with KNK437 and GEM was significant compared to GEM alone (one-way ANOVA). Furthermore, two-way ANOVA showed that this antitumor effect of the combined treatment was a synergistic effect ($p < 0.01$). Figure 4 shows the antitumor cytotoxic effect of KNK437 and GEM combined treatment on GEM-resistant PK59 cells. The antitumor effect of the combined treatment with KNK437 and GEM was not significant compared to GEM alone, but was significant compared to the untreated control (one-way ANOVA).

Discussion

HSP27 is a molecular chaperone that interacts with a large number of proteins and can prevent a wide variety of apoptotic agents from causing cell death. HSP27 belongs to the family of small HSPs, and it is expressed in virtually all organisms, from prokaryotes to mammals [19]. Garrido et al. [20] reported that HSP27 inhibited cytochrome c-dependent activation of procaspase-9 and prevented etoposide-induced apoptosis. Hansen et al. [21] reported that HSP27 altered the expression of topoisomerase II and inhibited doxorubicin-induced apoptosis in human breast cancer cells. Gibbons et al. [22] reported that overexpression of HSP27 in prostate cancer cells rendered cells resistant to both chemical- and radiation-induced apoptosis, which may be mediated by the production of survival factors. Urbani et al. [23] reported that the etoposide-resistant neuroblastoma cell clone showed overexpression of HSP27. These reports showed that the upregulation of antiapoptotic factors induced by HSP27 enhanced the resistance of tumor cells to chemotherapy. Furthermore, overexpression of HSP27 correlates with a poor prognosis and response to chemotherapy [24–26].

In our previous report, the expression of HSP 27 was increased in a GEM-resistant pancreatic cancer cell line, while HSP27-silenced cells showed increased sensitivity to GEM [6]. Furthermore, it has been clarified that upregulation of HSP27 in tumor specimens is related to higher resistance to GEM in patients with pancreatic cancer [6, 7]. These findings suggest that upregulation of HSP27 in advanced pancreatic cancer might contribute to GEM resistance, and silenced expression of HSP27 in GEM-resistant pancreatic cancer might increase the sensitivity to GEM. Thus, the present investigation was performed to prove that KNK437 downregulated the expression of HSP27 and increased GEM sensitivity. The results showed that KNK437 downregulated HSP27, and in vitro combined treatment with GEM significantly suppressed the growth of GEM-resistant pancreatic cancer cells. KNK437 is considered to be a possible candidate for combination therapy in anticancer drug regimes. Although KNK437 by itself did not show a cytotoxic effect on pancreatic cancer cells, it significantly increased the cytotoxic effect of GEM on cancer cells. This suggests that KNK437 could be used as a chemo-adjuvant to enhance the cytotoxic activity of GEM on pancreatic cancer cells.

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