Antitumor Effects of Eribulin Mesylate in Gemcitabine-resistant Pancreatic Cancer Cell Lines

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Abstract. *Background/Aim:* One reason of poor survival rate of patients with pancreatic cancer is the development of chemoresistance. The aim of the present study was to investigate the effects of eribulin mesylate in gemcitabine-refractory advanced pancreatic cancer cell lines. *Materials and Methods:* Three human pancreatic cancer cell lines (AsPC-1, Panc-1, and SUI-2) and human pancreatic endoderm (hPE) cells were used to evaluate the antitumor effects of gemcitabine and eribulin mesylate. Cell viability after treatment of cells with different concentrations of gemcitabine and eribulin mesylate was evaluated using water-soluble tetrazolium salts (WST) assays; cytotoxic effects were evaluated on the basis of morphological changes to cells. *Results:* Gemcitabine had no effect on cell viability of AsPC-1 nor Panc-1 cells, whereas gemcitabine reduced cell viability of SUI-2 cells in a dose-dependent manner. Eribulin mesylate significantly reduced cell viability of both AsPC-1 and Panc-1 cells ($p < 0.001$ and $p = 0.002$, respectively), but had no effect on hPE cells. Microscopic examination of AsPC-1 and Panc-1 cells after treatment with eribulin mesylate revealed morphological changes that included cell shrinkage, membrane blebbing, and fragmentation of the cells after drug exposure, and these were concentration-dependent effects. *Conclusion:* The findings of the present study suggest that eribulin mesylate may be a promising potential anticancer drug for gemcitabine-refractory advanced pancreatic cancer.

Pancreatic cancer is a commonly diagnosed cancer worldwide, with an extremely poor prognosis, and is the fourth leading cause of cancer-related deaths (1). In patients with recurrent, metastatic, or advanced pancreatic cancer, chemotherapy can prolong survival and improve quality of life compared with best supportive care (2, 3). In recent years, gemcitabine, a novel pyrimidine nucleoside analog, has become the standard chemotherapeutic agent used in patients with pancreatic cancer (3). Despite considerable efforts to improve outcomes for these patients, response rates to chemotherapy remain low and the median survival of patients with advanced disease remains in the range of 6 to 10 months (3, 4). Therefore, more effective drugs or combination therapies for pancreatic cancer are needed.

Eribulin mesylate, a completely synthetic macrocyclic ketone analog of halichondrin B, which is a natural large polyether macrolide from a rare marine Japanese sponge, acts as a tubulin-binding agent, interfering with microtubule dynamics and suppressing microtubule polymerization without affecting depolymerization (5, 6). Although eribulin mesylate is a novel drug that has shown significant activity and efficacy in heavily pretreated patients with refractory breast cancer, its efficacy in the treatment of pancreatic cancer in patients with a poor prognosis is not clear (7). In the present study, we investigated the antiproliferative effects of eribulin mesylate in three different pancreatic cancer cell lines.

Materials and Methods

Materials and reagents. The present study was performed on three human pancreatic cancer cell lines (AsPC-1, Panc-1, and SUI-2) and human pancreatic endoderm (hPE) cells. The AsPC-1 and Panc-1 cell lines were purchased from the European Collection of Authenticated Cell Cultures (Salisbury, Wiltshire, UK), the SUI-2 cell line was purchased from the Japan Health Sciences Foundation (Tokyo, Japan), and the hPE cell line was purchased from Cell Systems Corporation (Kirkland, WA, USA). Dulbecco's modified Eagle's medium (DMEM) and antibiotics (penicillin, streptomycin) were purchased from Invitrogen Corporation (New York, NY, USA). Stock solutions of both eribulin mesylate (Halaven powder; Eisai

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Co. Ltd, Tokyo, Japan) and gemcitabine (Gemzar powder; Eli Lilly Co. Ltd, Tokyo, Japan) were created by diluting the drugs in water to a concentration of 0.5 µg/µl; stock solutions were stored at -20°C until use. Fetal serum albumin (FBS) was obtained from Sigma Chemical (St. Louis, MO, USA).

Cell culture. The three pancreatic cancer cell lines and hPE cells were cultured in DMEM supplemented with penicillin, streptomycin, and 8% FBS, in a 95% air/5% CO₂ atmosphere at 37°C in a humidified incubator.

Determination of cell viability. The antiproliferative activity of test solutions was determined using water-soluble tetrazolium salts (WST) assays in accordance with the manufacturer's instructions. Briefly, 5×10³ cells/well were seeded onto 96-well plates and incubated at 37°C, under 5% CO₂, for 24 h. Different concentrations of gemcitabine (5, 10, 50 ng/ml) or eribulin (0.5, 1, 5, 10 ng/ml) were then added to each well, and cells were incubated for a further 90 min at 37°C. The respective concentrations of each drugs used were based on previous *in vitro* and *in vivo* studies (5-9). As a control, cells were incubated with saline under the same conditions. At the end of the 90-min incubation period, water-soluble tetrazolium salts (WST) reagent (Sigma-Aldrich, Tokyo, Japan) was added to each well and the cells were incubated for another 90 min at 37°C. Absorbance was then measured with a microplate absorbance reader (Molecular Devices, Sunnyvale, CA, USA) using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA) at 450 nm with a reference wavelength of 620 nm.

Morphological observations. To evaluate morphological changes caused by cytotoxic effects, all cell lines were cultured in a 10-cm dish at a density of 4×10⁵ cells/ml in a humidified incubator at 37°C under 5% CO₂. Gemcitabine (5, 10, 50 ng/ml) or eribulin mesylate (0.5, 1, 5, 10 ng/ml) was added to each dish, and cells were incubated for 24 h at 37°C. Cells were then observed under a fluorescence microscope (BZ-9000; Keyence, Itasca, IL, USA) to analyze morphological changes.

Statistical analysis. To determine the significance of differences between two groups under multiple conditions, data were analyzed by one-way analysis of variance, followed by Bonferroni multiple comparison tests. All statistical analyses were performed using PRISM statistical analysis software (GraphPad Software Inc., San Diego, CA, USA). A two-sided *p*-value of less than <0.05 was considered significant.

Results

Effects of gemcitabine on cell viability. The cell viability of the control group was set at 100% and the effects of gemcitabine on cell viability are expressed relative to the control group (Figure 1). At 5, 10, and 50 ng/ml, gemcitabine significantly reduced the cell viability of SUIT-2 cells in a dose-dependent manner (*p*<0.001; Figure 1a). However, although significant dose-dependent effects were seen for 5, 10, and 50 ng/ml gemcitabine on AsPC-1 (*p*=0.005; Figure 1b) and Panc-1 cells (*p*=0.046; Figure 1c), the cell viability of these two cell lines did not differ significantly from that of the control group. Thus, these two cell lines are considered gemcitabine-refractory.

In addition, although gemcitabine exhibited significant dose-dependent effects on hPE cells (*p*=0.043), there was no significant difference in cell viability between control and gemcitabine-treated hPE cells (Figure 1d).

Effects of eribulin mesylate on cell viability. The cell viability of the control group was set at 100% and the effects of eribulin mesylate on cell viability are expressed relative to the control group (Figure 2). At 0.5, 1, 5, and 10 ng/ml eribulin mesylate significantly and dose-dependently reduced the cell viability of both AsPC-1 (*p*<0.001; Figure 2a) and Panc-1 cells (*p*=0.002; Figure 2b). Eribulin mesylate had no significant effect on the cell viability of hPE cells at these concentrations (Figure 2c).

Morphological changes. Figure 3 shows representative images of the morphological changes seen following the exposure of AsPC-1 and Panc-1 cells to different concentrations of eribulin mesylate in three independent experiments. Tumor cells from both lines exhibited morphological changes after eribulin mesylate treatment that included cell shrinkage, membrane blebbing, and cell fragmentation. These effects increased with increasing dose of eribulin mesylate. The antiproliferative effects of eribulin mesylate were dose-dependent for both gemcitabine-refractory human pancreatic cancer cell lines.

Discussion

The results of the present study demonstrated that eribulin mesylate reduces the cell viability of human pancreatic cancer cell lines that are resistant to gemcitabine; these findings were confirmed by morphological analysis and, together, the data indicate inhibition of tumor cell proliferation by eribulin mesylate. To the best of our knowledge, the present study is the first to report on the anticancer effects of eribulin mesylate in gemcitabine-refractory advanced pancreatic cancer cell lines. The findings of the present study suggest that eribulin mesylate has significant potential in the treatment of advanced pancreatic cancer.

Gemcitabine has become the standard treatment for patients with unresectable pancreatic cancer, improving patient survival compared with fluorouracil (2-4,10). However, the prognosis for patients with pancreatic cancer remains poor because of the rapidly progressive and aggressive nature of the disease. Indeed, the median progression-free survival has been reported to be in the range of 2-6 months, or even less in the setting of gemcitabine resistance, despite recent advances in the understanding of the underlying biology of pancreatic cancer and improvements in imaging modalities (3, 4, 11).

Based on the results of the present study, the pancreatic cancer cell lines could be classified into two groups: gemcitabine-sensitive and -resistant groups. The resistance

Figure 1. Cell viability in the SUIT-2 (a), AsPC-1 (b), and Panc-1 (c) pancreatic cell lines, as well as of human pancreatic endoderm (hPE) cells (d), with and without gemcitabine treatment, as determined using water-soluble tetrazolium salt assays. Cell viability was set at 100% in the control group (no drug treatment) and viability after gemcitabine treatment is shown as a percentage of that in the control group. Data are the mean $\pm$ SD of experiments performed in triplicate. *Significantly different from the respective control ($p < 0.05$).

Figure 2. Cell viability in the AsPC-1 (a) and Panc-1 (b) pancreatic cell lines, as well as human pancreatic endoderm (hPE) cells (c), with and without eribulin mesylate treatment, as determined using water-soluble tetrazolium salt assays. Cell viability was set at 100% in the control group (no drug treatment) and viability after eribulin mesylate treatment is shown as a percentage of that in the control group. Data are the mean $\pm$ SD of experiments performed in triplicate. *Significantly different from the respective control ($p < 0.05$).

Figure 3. Morphological changes in AsPC-1 and Panc-1 cells, as observed by fluorescence microscopy, after 24 h exposure to 0.5 or 10 ng/ml eribulin mesylate. Control cells were exposed to saline, instead of drug, for 24 h. Morphological changes observed after eribulin mesylate treatment included cell shrinkage, membrane blebbing, and cell fragmentation. The photographs shown are representative images of three independent experiments.

of pancreatic cancer to chemotherapeutic reagents appears to be related to various factors, including nucleoside transport and metabolism, cell-cycle regulation, proliferation, and apoptosis; in addition, multiple gene products are linked to chemoresistance. For example, Akada *et al.* identified a number of genes that are potentially involved in intrinsic resistance to gemcitabine in pancreatic cancer cell lines, reporting that B-cell lymphoma 2 (BCL2)/adenovirus E1B 19 kDa protein interacting protein and the phosphatidylinositol 3-kinase/protein kinase B pathway may play an important role in poor sensitivity to gemcitabine chemotherapy (12). These molecular markers may be promising candidate tools for the detection of gemcitabine chemosensitivity.

Even if patients with pancreatic cancer show initial sensitivity to gemcitabine, development of resistance is common. Therefore, agents that can be used instead of gemcitabine or to overcome gemcitabine resistance are needed (5, 13). In Japan, S-1, a combination of tegafur, gimeracil, and oteracil potassium, has been developed as an anticancer drug for the treatment of pancreatic cancer. Various clinical trials using S-1 have demonstrated promising activity not only against gastrointestinal cancer, including gastric cancer and colorectal cancer, but also pancreatic cancer (4, 14, 15). S-1 monotherapy is accepted as an alternative treatment option for unresectable pancreatic cancer because it is well tolerated and is associated with lower hematological and gastrointestinal toxicities (4). Furthermore, it was expected that the combined administration of S-1 and gemcitabine may be

more effective, because the median overall survival for patients with unresectable pancreatic cancer in the gemcitabine, S-1, and combination groups was reported to be 7.5, 8.0, and 10.5 months, respectively (4).

Eribulin mesylate has a unique interaction with tubulin, and this may help overcome the resistance mechanisms that limit the effectiveness of other agents (16). Furthermore, eribulin mesylate may have a role in inactivating BCL2 family proteins, which are mediators of chemoresistance in pancreatic cancer (7, 12). Recently, it has been demonstrated that the epithelial–mesenchymal transition (EMT) is a crucial process that is often activated during cancer invasion and metastasis and also induces chemoresistance in cancer treatment (17, 18). It has been reported that eribulin mesylate has significant effects on EMT-related pathways, resulting in increased antitumor effects (19, 20). The present study clearly shows that eribulin mesylate has antitumor effects, namely reducing cell viability and inducing morphological changes in tumor cells, which could be beneficial in clinical therapy for gemcitabine-refractory advanced pancreatic cancer. Because neither gemcitabine nor eribulin mesylate had any effect on the cell viability of hPE cells, which were derived from human pancreatic endoderm cells, at the concentrations tested, it may be that these concentrations of gemcitabine and eribulin mesylate will prove to be safe for use in humans.

The present study does have some limitations. Firstly, the results reported for the preliminary cytotoxicity tests cannot be extrapolated directly to clinical situations because laboratory studies cannot simulate the complex biological conditions seen in the clinical setting. Recent phase I and II

clinical studies reported that eribulin mesylate was well-tolerated and exhibited anticancer activity in patients with advanced solid tumors (21, 22). However, randomized control trials performed on a large number of patients are required to confirm the survival benefit of eribulin mesylate for advanced pancreatic cancer.

In conclusion, the findings of the present study, together with those of previous studies, suggest that eribulin mesylate is a cytotoxic agent that could be developed for use as an anticancer drug for the treatment of gemcitabine-refractory advanced pancreatic cancer. Further studies, including *in vivo* studies in animal models and clinical randomized control trials, are needed to establish the benefits of and responses to this agent in patients with advanced pancreatic cancer that is resistant to gemcitabine.

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Conflicts of Interest

None.

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