The HSP70 and autophagy inhibitor pifithrin-μ enhances the antitumor effects of TRAIL on human pancreatic cancer

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Running title: Pifithrin-μ enhances the TRAIL-induced antitumor effects

Keywords: TRAIL, pancreatic cancer, pifithrin-μ, HSP70, autophagy

Abbreviations list:

AIF, apoptosis-inducing factor; DR, death receptor; PFT, pifithrin; PI, propidium iodide; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

Financial support: This study was supported in part by Grants-in aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (no. 18591449 to M. Harada, no. 23701074 to N. Harashima), an from Shimane University "S-TAKUMI Medical Nanotechnology" Project (to M. Harada).

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Conflicts of interest: None

The word count (excluding references): 4997 words

6 Figures and no Table

Abstract

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and agonistic death receptor-specific antibodies can induce apoptosis in cancer cells with little cytotoxicity to normal cells. To improve TRAIL-induced antitumor effects, we tested its effectiveness in combination with pifithrin-μ, which has the potential to inhibit HSP70 function and autophagy, both of which participate in TRAIL resistance in cancer cells. Among the four human pancreatic cancer cell lines tested, MiaPaca-2, Panc-1, and BxPC-3 cells showed varying sensitivities to TRAIL. In MiaPaca-2 and Panc-1 cells, knockdown of HSP70 or Beclin-1, the latter an autophagy-related molecule, by RNA interference augmented TRAIL-induced antitumor effects, decreasing cell viability and increasing apoptosis. Based on these findings, we next determined whether the TRAIL-induced antitumor effects could be augmented by its combination with pifithrin-μ. The combination of TRAIL plus pifithrin-μ significantly decreased the viability and colony-forming ability of MiaPaca-2 and Panc-1 cells compared to cells treated with either agent alone. When applied alone, pifithrin-μ increased Annexin V^+ cells in both caspase-dependent and caspase-independent manners. It also promoted TRAIL-induced apoptosis and arrested cancer cell growth. Furthermore, pifithrin-μ antagonized TRAIL-associated NF-κB activation in cancer cells. In a xenograft mouse model, combination therapy significantly

inhibited MiaPaca-2 tumor growth compared to treatment with either agent alone. The results of this study suggest protective roles for HSP70 and autophagy in TRAIL resistance in pancreatic cancer cells and suggest that pifithrin-μ is a promising agent for use in therapies intended to enhance the antitumor effects of TRAIL.

Introduction

Members of the tumor necrosis factor (TNF) cytokine family, such as TNF-α and the Fas ligand, play important roles in inflammation and immunity (1). However, their use in anti-cancer therapy is limited because they cause severe cytotoxicity to normal cells. In contrast, TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, can induce apoptosis in cancer cells while causing almost no cytotoxicity to normal cells (2). Therefore, TRAIL has been applied for several clinical trials (3, 4). TRAIL binds to death receptors (DRs) and activates caspases, leading to apoptosis. However, some cancer cells acquire TRAIL resistance (5). The binding of TRAIL to DRs activates NF-κB, PKC, MAPK, and AKT signaling (6, 7), thereby promoting cell proliferation and apoptosis resistance. Notably, activation of NF-κB signaling is an important TRAIL resistance mechanism (6, 8). Indeed, combination therapy with TRAIL plus NF-κB inhibitors has been reported to be a promising treatment strategy (9-11).

Cancer cells acquire therapy resistance through a variety of mechanisms. One is increased expression of HSP70 (12). In contrast to the very low level in unstressed normal cells, HSP70 expression is rapidly increased in response to various stresses (13, 14). Importantly, increased expression of HSP70 in cancer cells has been reported to be associated with malignant features and poor prognosis in cancer patients (15). Autophagy, meanwhile, has received much attention as a mechanism of therapy resistance in cancer cells. Its fundamental role in cells is cytoprotection under starvation and stress conditions (16) but this protective function can render cancer cells therapy-resistant (17). Indeed, many reports suggest that inhibition of autophagy in cancer cells can restore their susceptibility to anti-cancer therapy (18-20). Recently, we reported that autophagy inhibits apoptosis of human prostate and breast cancer cells treated with an innate adjuvant receptor ligand (21, 22). These lines of evidence suggest that HSP70 and autophagy are promising targets for therapies intended to enhance TRAIL-induced antitumor effects, something that has not yet been fully investigated.

Pifithrin (PFT)-μ (2-phenylethynesulfonamide) was initially identified as a small-molecule inhibitor of the binding of p53 to mitochondria (23). Recently, it was revealed to interact selectively with HSP70 and to inhibit its functions (24). Additionally, it induces altered autophagy, leading to inhibition of the later autophagic pathway. Furthermore, PFT-μ inhibits the degradation of IκBα, resulting in inhibition of the NF-κB pathway. This information led us to test the possibility that PFT-μ enhances TRAIL-induced antitumor effects in human pancreatic cancer. In this study, after confirming that HSP70 and autophagy play protective roles in TRAIL-induced antitumor effects, we demonstrated that the combination of TRAIL and PFT-μ decreased the viability and colony-forming ability of pancreatic cancer cells, and increased their cell death. Using a xenograft mouse model, we also showed that the combination therapy significantly decreased pancreatic tumor growth compared to treatment with either agent alone. These results suggest that PFT-μ is a promising enhancer of TRAIL-induced antitumor effects on human pancreatic cancer.

Materials and Methods

Cell lines. Four human pancreatic cancer cell lines (MiaPaca-2, Panc-1, AsPC-1, and BxPC-3), which were kindly provided by Dr. K. Takenaga (Shimane University Faculty of Medicine), were maintained in DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS (Invitrogen, Grand Island, NY, USA) and 20 μg/ml gentamicin (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO_2. No authentication was done by the authors.

Cell viability assay. Cell viability was evaluated using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay (Nacalai Tesque, Kyoto, Japan). Briefly, cells were seeded in flat-bottomed 96-well plates. The next day, TRAIL (PeproTech Inc., Rocky Hill, NJ, USA) and/or PFT-μ (Santa Cruz Biotechnology (SCB), Santa Cruz, CA, USA, or Cayman Chemical, Ann Arbor, MI, USA) were added. Two days later, WST-8 was added to each well, and the plates were read at a wavelength of 450 nm after 3 h. For inhibition assays, z-VAD-fmk (R&D Systems, Minneapolis, MN, USA) was added, and DMSO was used as a vehicle control.

Flow cytometry. Cell death was measured using the Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, CA, USA) and propidium iodide (PI). To examine the cell cycle and proliferation of cancer cells, a BrdU/7AAD Prolifesration Kit (Becton Dickinson, Fullerton, CA, USA) was used according to the manufacturer's instructions. Analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson).

Immunoblot. Cells were lysed with a mammalian protein extraction reagent (M-PER; Thermo Scientific, Rockford, IL, USA) containing a protease inhibitor cocktail (Nacalai Tesque). Equal amounts of protein were resolved on 4–12% gradient or 12% SDS-PAGE gels, and then transferred to polyvinylidene fluoride membranes. The membranes were blocked and the blots then incubated with the following primary antibodies: anti-LC3 (MBL), anti-cyclinD1 (Cell Signaling Technology (CST), Danvers, MA, USA), anti-c-Myc (Epitomics, Burlingame, CA, USA), anti-Beclin-1 (CST), anti-Cathepsin L (SCB), anti-IκBα (CST), anti-β-actin (BioLegend, San Diego, CA, USA), and anti-α-tubulin (SCB). Goat anti-rabbit and goat anti-mouse alkaline phosphatase-conjugated secondary antibodies (Invitrogen) were used to detect the primary antibodies.

Transfection of small interfering RNA (siRNA). Transfection of siRNA was performed using Lipofectamine™ RNAiMAX (Invitrogen), according to the manufacturer's instructions. HSP70 siRNA (sc-29357) and Beclin-1 siRNA (sc-29797) were purchased from SCB. Control siRNA (#6568) was purchased from CST. Three days after siRNA transfection, cancer cells were used for subsequent experiments.

Confocal imaging. LC3B (NM_022818) was amplified by PCR and inserted into the pcDNA3.1/NT-GFP-TOPO vector (Invitrogen) in frame with the GFP sequence. Transfection of plasmids was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cells were cultured on round microscope cover glasses in 24-well plates with the indicated reagents for 2 days. After incubation with Hoechst 33342 (5 μg/ml) for 30 min, cells were fixed with 3% formalin and placed on slide glasses with 4 μl of mounting medium for fluorescence (Vectashield; Vector Laboratories, Inc., Burlingame, CA, USA). To examine NF-κB translocation to the nucleus, cells were cultured on round microscope cover glasses in 24-well plates for one day. After incubation with PFT-μ (20 μM) for 5 h, cells were additionally cultured in the presence of TRAIL and Hoechst 33342 (5 μg/ml) for 30 min. After fixation and permeabilization with 3% formalin and 1% Triton X, respectively, cells were stainined with anti-NF-κB p65 antibody (CST), followed by Alexa Fluor 488-conjugated anti-rabbit IgG $F(ab')_2$ fragment (CST). Confocal imaging was performed using an Olympus FV1000-D laser scanning microscope (Olympus, Tokyo, Japan).

Colony-forming assay. Cancer cells were seeded in six-well plates in the presence or absence of TRAIL and/or PFT-μ. Two days later, the medium was replaced with medium that contained no reagent, and the culture was continued for an additional 10 days. Thereafter, colonies were counted after fixation with methanol and staining with 0.05% crystal violet.

***In vivo* xenograft model.** BALB *nu/nu* female mice, purchased from CLEA Japan Inc. (Tokyo, Japan), were maintained under specific-pathogen-free conditions. Experiments were performed according to the ethical guidelines for animal experimentation of the Shimane University Faculty of Medicine (approval number: IZ24-5). Mice were inoculated in the right flank with 4×10^6 MiaPaca-2 cells with Matrigel (Japan BD Biosciences, Tokyo, Japan) at a volume ratio of 1:1. On day 20, the mice were pooled and divided into four groups. On days 1, 2, and 3 after grouping, the mice were injected intraperitoneally with PFT-μ (100 μl). As a vehicle control, 100 μl of DMSO was injected. On day 2 after grouping, the mice were injected once intratumorally with TRAIL (50 μl). As a vehicle control, 50 μl of culture medium was injected. Thereafter, tumor size was measured twice weekly.

Statistical analyses. Data were evaluated statistically using an unpaired two-tailed

Student's *t*-test or ANOVA with Scheffe's *post hoc* test. A *P* value of less than 0.05 was considered to indicate statistical significance.

Results

Protective role of HSP70 in TRAIL-induced antitumor effects on pancreatic cancer cells

First, we examined the expression of DR4 and DR5 on four pancreatic cancer cell lines. Although the expression of DR4 was relatively low on three cell lines and was negative on Panc-1, all cell lines were strongly positive for DR5 (Suppl. Fig. 1). We next determined the TRAIL susceptibility of these cell lines (Fig. 1A). MiaPaca-2, BxPC-3, and Panc-1 cells were highly, moderately, and lowly sensitive to TRAIL, respectively, and their susceptibility was dose-dependent. In contrast, AsPC-1 cells were entirely resistant to TRAIL. In subsequent experiments, we focused on MiaPaca-2 and Panc-1 cells.

To elucidate the roles of HSP70 in TRAIL-induced antitumor effects on human pancreatic cancer cells, we compared the sensitivities of MiaPaca-2 and Panc-1 cells in which HSP70 expression was blocked by RNA interference (Fig. 1B). Knockdown of HSP70 decreased the viability of MiaPaca-2 and Panc-1 cells treated with TRAIL (Fig. 1C). Knockdown of HSP70 markedly increased the percentage of Annexin V^+ Panc-1, but not

MiaPaca-2, cells upon TRAIL treatment (Fig. 1 D). As shown in Fig. 1E, knockdown of HSP70 increased Annexin V^{+}/PI^{-} early apoptotic and Annexin V^{+}/PI^{+} late apoptotic and/or necrotic Panc-1 cells upon TRAIL treatment. A notable finding is that Panc-1 cells, which were less sensitive to TRAIL, became sensitive to low-dose TRAIL after knockdown of HSP70. Overall, these results indicate that HSP70 plays a protective role in TRAIL-induced antitumor effects on human pancreatic cells.

Protective role of autophagy in TRAIL-induced antitumor effects on pancreatic cancer cells

We next tested the possibility that autophagy plays a protective role in the TRAIL resistance of pancreatic cancer cells. LC3 exists in two forms: LC3-type I, which is cytosolic, and its proteolytic derivative, LC3-type II, which localizes to the autophagosomal membrane (25). As shown in Fig. 2A, TRAIL treatment increased the expression of LC3-type II in MiaPaca-2 cells, while LC3-type II was expressed in Panc-1 cells without TRAIL treatment. We also assessed autophagy by confocal imaging of LC3 foci in GFP-LC3 fusion protein-expressing cancer cells (Fig. 2B). These two cell lines were transiently transfected with a plasmid encoding GFP-LC3, and GFP-LC3 foci were subsequently examined. No GFP-LC3 foci were detected in control GFP/NT-transfected MiaPaca-2 and Panc-1 cells. Although GFP-LC3 foci were detected in

GFP-LC3-transfected MiaPaca-2 and Panc-1 cells, even without TRAIL treatment, GFP-LC3 foci appeared to increase in both number and size after TRAIL treatment. To elucidate the role of autophagy in TRAIL-induced antitumor effects, the expression of Beclin-1, a molecule required for autophagy, was knocked down by transfection of Beclin-1 siRNA (Fig. 2C). Knockdown of Beclin-1 decreased the viabilities and increased the percentages of Annexin V^+ MiaPaca-2 and Panc-1 cells upon TRAIL treatment (Fig. 2 D and E). Collectively, these results indicate that, similarly to HSP70, autophagy plays a protective role in TRAIL-induced antitumor effects on pancreatic cancer cells.

Antitumor effects on pancreatic cancer cells of the combination of TRAIL and PFT-μ

Since both HSP70 and autophagy were suggested to function protectively upon TRAIL treatment of pancreatic cancer cells, we next determined whether TRAIL-induced antitumor effects were enhanced by PFT-μ, which inhibits both the functions of HSP70 and autophagy (24), as described in the Introduction. Fig. 3A shows the structure of PFT-μ. As shown in Fig. 3B, when administered alone, PFT-μ dose-dependently decreased the viability of all four pancreatic cancer cell lines. When suboptimal doses of TRAIL and PFT-μ were combined, additive effects were evident in three cell lines other than AsPC-1 (Fig. 3C). Combined treatment with suboptimal doses of TRAIL and PFT-μ significantly decreased the colony-forming ability of MiaPaca-2 and Panc-1 cells compared to those treated with either agent alone (Fig. 3D). Since the continuous presence of PFT-μ for 12 days drastically inhibited colony formation, we cultured cancer cells for 2 days with TRAIL and/or PFT-μ and subsequently for 10 days without reagents.

PFT-μ induces cell death and growth arrest of pancreatic cancer cells

In the studies described above, we examined the antitumor effects on pancreatic cancer cells by measuring viability 2 days after the administration of TRAIL and/or PFT-μ. However, such effects on viability may reflect alterations in cell death and/or growth. Therefore, we next examined the underlying mechanism of action of PFT-μ in detail. Although suboptimal doses of TRAIL slightly increased Annexin V^+ MiaPaca-2 and Panc-1 cells, PFT-μ moderately increased the percentages of Annexin V^+ cells (Fig. 4A). Combining PFT-μ with TRAIL further increased the number of Annexin V^+ cells. Since PFT-μ was reported to induce caspase-independent apoptosis (24), we attempted to confirm the result in our experimental systems. TRAIL-induced increase of Annexin V^+ MiaPaca-2 and Panc-1 cells was completely inhibited by the pan-caspase inhibitor z-VAD, while z-VAD rescued in part PFT-μ–induced cell death (Fig. 4B). This result indicates that PFT-μ increased Annexin V^+ cells in both caspase-dependent and caspase-independent manners. We next determined whether growth arrest was involved in the antitumor effects of PFT-μ by evaluating BrdU uptake and 7AAD staining. PFT-μ significantly decreased the percentages of $BrdU^+$ S-phase MiaPaca-2 and Panc-1 cells (Fig. 4C and D) and increased the apoptotic sub-G1 fraction in MiaPaca-2 cells. We also examined the expression of proliferation-related proteins in MiaPca-2 and Panc-1 cells, and found that PFT-μ decreased cyclin-D1 expression in Miapaca-2 cells but not Panc-1 cells. Collectively, these results indicate that PFT-μ induces two types of antitumor responses in pancreatic cancer cells: cell death, both caspase-dependent and caspase-independent, and cell-growth arrest.

PFT-μ inhibits the autophagy-lysosomal system and TRAIL-associated NF-κB activation in pancreatic cancer cells

We next attempted to elucidate the mechanisms by which PFT-μ enhanced TRAIL-induced antitumor effects. To evaluate the influence of PFT-μ on the autophagy system, we determined its effect on the degradation of pro-Cathepsin L (Fig. 5A). PFT-μ increased the expression of LC3 type II. However, this increase was not the result of enhanced autophagy, but rather inhibition of the autophagy-lysosomal system, because degradation of pro-Cathepsin L to Cathepsin L was clearly inhibited, as reported previously (24). We also examined the effect of PFT-μ on NF-κB signaling in TRAIL-treated cancer cells, because activation of NF-κB is a major mechanism of TRAIL resistance of cancer cells (6, 8). As expected, TRAIL significantly decreased the level of IκBα, indicating activation of the NK-κB pathway. Although the IκBα level was decreased in cells treated with PFT-μ alone, PFT-μ restored IκBα expression in cells treated with TRAIL (Fig. 5B). TRAIL and PFT-μ had no marked effect on HSP70 expression in cancer cells. We next directly confirmed that the combination treatment inhibited the NF-κB pathway using confocal imaging. Although the treatment with either of TRAIL or PFT-μ induced NF-κB translocation to the nucleus, the combination treatment decreased the expression of NF-κB in the nucleus (Fig. 5C). These results suggest that PFT-μ can inhibit the autophagy-lysosomal system and antagonize TRAIL-associated NF-κB activation in pancreatic cancer cells.

***In vivo* antitumor effect of TRAIL plus PFT-μ combination therapy in a xenograft mouse model**

Finally, we evaluated whether combination therapy with TRAIL plus PFT-μ exerted an antitumor effect against established human pancreatic cancer in a xenograft mouse model. Nude mice were inoculated with MiaPaca-2 cells and were grouped when tumor diameters reached 8–9 mm. After grouping, PFT-μ (25 mg/kg) was injected intraperitoneally on days 1, 2, and 3 and TRAIL was injected locally on day 2. No change in body weight resulted, suggesting no severe adverse event (data not shown). Although the systemic administration of PFT-μ had no antitumor effect and local injections of TRAIL decreased tumor growth moderately, but not significantly, combination therapy with TRAIL plus PFT-μ significantly suppressed tumor growth (Fig. 6A and B).

Discussion

Since pancreatic cancer is highly resistant to conventional anti-cancer therapies and is associated with a very poor prognosis (26), new treatment modalities to enhance the efficacy of current treatments are required. In this study, we investigated the possibility that PFT-μ, a small-molecule HSP70 inhibitor that has the ability to alter autophagy (24), could enhance TRAIL-induced antitumor effects on human pancreatic cancer cells. We found that both HSP70 and autophagy are, at least in part, responsible for the TRAIL resistance of cancer cells, and that PFT-μ enhances TRAIL-induced antitumor effects on human pancreatic cancer cells.

HSP70 is a potent heat-inducible survival protein that confers cytoprotection against various death-inducing stimuli and increases tumorigenicity (27-29). It has been suggested to be a promising target in cancer treatment (30). In this study, knockdown of HSP70 significantly decreased cell viability and increased the percentage of Annexin V^+ pancreatic cancer cells after TRAIL treatment (Fig. 1). The increase of Annexin V^+ cells in response to TRAIL was stronger in the less TRAIL-sensitive Panc-1 cells than in highly TRAIL-sensitive MiaPaca-2 cells. Although only one report suggests that TRAIL-induced apoptosis in cancer cells is enhanced by HSP70 (31), many reports have shown that HSP70 inhibits TNF-α- and Fas-mediated apoptosis in cancer cells (32-34). In this study, we demonstrated that siRNA knockdown of HSP70 increased Annexin V^+ Panc-1 cells after TRAIL treatment (Fig. 1). To our knowledge, this is the first report that HSP70 contributes to the TRAIL resistance of human pancreatic cancer cells. Another intriguing finding is that autophagy is protective in TRAIL-treated pancreatic cancer cells. This result is compatible with other reports (20). Autophagy has received much attention in various cell biology fields (16, 17). Despite the reports of autophagic cell death (35-37) and autophagy-dependent antitumor immune response (38), the fundamental role of autophagy is thought to be cytoprotection under starvation and stress conditions (16). Many reports suggest that autophagy functions cytoprotectively in cancer cells (19, 20, 39, 40). In line with this, we previously reported that autophagy protects against apoptosis in human prostate and breast cancer cells after treatment with an innate adjuvant receptor ligand (21, 22). Therefore, in this study, we investigated the participation of autophagy in the TRAIL resistance of pancreatic cancer cells. Autophagy was constitutively induced in MiaPaca-2 and Panc-1 cells, and TRAIL treatment apparently augmented autophagy in MiaPaca-2 cells. We also showed that autophagy inhibition by knockdown of Beclin-1 increased the susceptibility of pancreatic cancer cells to TRAIL (Fig. 2). Our findings regarding the protective roles of HSP70 and autophagy in TRAIL treatment encouraged us to utilize the

HSP70 and autophagy inhibitor PFT-μ to enhance TRAIL-induced antitumor effects on pancreatic cancer cells.

DR-mediated TRAIL signaling is involved in activation of the NF-κB pathway, leading to TRAIL resistance (6, 8). Indeed, the proteasome inhibitor bortezomib enhances TRAIL-induced antitumor effects by inhibiting NF-κB (9-11). Interestingly, PFT-μ decreases IκBα degradation by inhibiting the autophagy-lysosomal system (24). PFT-μ also inhibits the proteasome system (41). We then determined whether PFT-μ inhibited TRAIL-induced activation of the NF-κB pathway. As expected, TRAIL decreased IκBα expression in cancer cells, and co-treatment with PFT-μ inhibited IκBα degradation. In addition, confocal imaging revealed that the combination treatment inhibited NF-κB translocation to the nucleus. This may be the third mechanism by which PFT-μ augmented TRAIL-induced antitumor effects on pancreatic cancer cells.

How does HSP70 inhibit TRAIL-induced cell death? HSP70 interacts with Apaf-1 to prevent caspase activation (42, 43). Interestingly, HSP70 has been reported to localize to the membranes of lysosomes, promote cancer cell viability, and inhibit TNF-induced cell death by inhibiting lysosomal membrane permeabilization (44). Thus HSP70 enhances survival by stabilizing the lysosomes in cancer cells. Since PFT-μ binds HSP70, inhibiting the autophagy–lysosomal system, it is possible that PFT-μ inhibits HSP70-induced stabilization of lysosomal membrane permeabilization, resulting in increased cell death. We plan to investigate this possibility.

HSP70 can bind to apoptosis-inducing factor (AIF), which induces caspase-independent apoptosis by translocation into the nucleus (45). As PFT-μ induces cell death in both caspase-dependent and -independent manners in cancer cells (Fig. 4B), we evaluated the role of AIF in PFT-μ–induced caspase-independent cell death. However, RNA interference of AIF had no effect on PFT-μ–induced cell death of pancreatic cancer cells (data not shown); thus, AIF likely does not participate in PFT-μ–induced cell death.

There are several possible explanations as to how autophagy protects cancer cells from TRAIL-induced cell death. First, autophagy degrades caspases. Indeed, autophagy can degrade active caspase 8 (46), which is required for extrinsic signal-mediated caspase-dependent apoptosis. Second, although we can not exclude the possibility of participation of the proteasome system, the autophagy–lysosomal system degrades IκBα, resulting in activation of the NF-κB pathway. Several reports have indicated that activation of this pathway can cause cancer cells to become resistant to TRAIL (6, 8). Interestingly, a recent report suggests that the mitochondrial tumor suppressor ARF interacts with HSP70, and that PFT-μ can reduce the ability of ARF to induce autophagy by selectively interacting with HSP70 and blocking ARF trafficking to mitochondria (47). ARF thus seems to play a crucial role in the altered autophagy induced by PFT-μ. We plan to elucidate this mechanism in more detail.

Besides cell death, PFT-μ induced growth arrest of pancreatic cancer cells. PFT-μ treatment significantly decreased the S-phase fraction in two cancer cell lines (Fig. 4C). The decreased cyclin D1 expression might, at least in part, account for PFT-μ–induced cell growth arrest. We performed a long-term (12-day) colony-formation assay. A suboptimal dose of PFT-μ significantly decreased the colony-forming ability of MiaPaca-2 cells. These data support the hypothesis that PFT-μ arrests cell growth.

Among the four pancreatic cancer cell lines, only AsPC-1 was resistant to TRAIL, and PFT-μ showed no sensitizing effects on AsPC-1. Thus some TRAIL susceptibility appears to be necessary for the sensitizing effects of PFT-μ. Additionally, only AsPC-1 was entirely TRAIL-resistant even though there was no difference in DR4 and DR5 expression among the four cell lines (Suppl. Fig. 1). Although we have not elucidated the underlying mechanisms, AsPC-1 may have been preferentially positive for nonfunctional decoy receptors or anti-apoptotic molecules including bcl-2, bcl-xL, and c-FLIP, as reported previously (48, 49).

In conclusion, we show here that PFT-μ has the potential to induce two types of antitumor response; *i.e*., cell death and cell-growth arrest, in human pancreatic cancer cells, and that it enhances TRAIL-induced antitumor effects both *in vitro* and *in vivo*. These results suggest that PFT-μ shows promise for use as a therapy intended to enhance the antitumor effects of TRAIL or agonistic antibodies against DRs.

Acknowledgements: We thank Ms. Tamami Moritani for her technical assistance.

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Figure legends

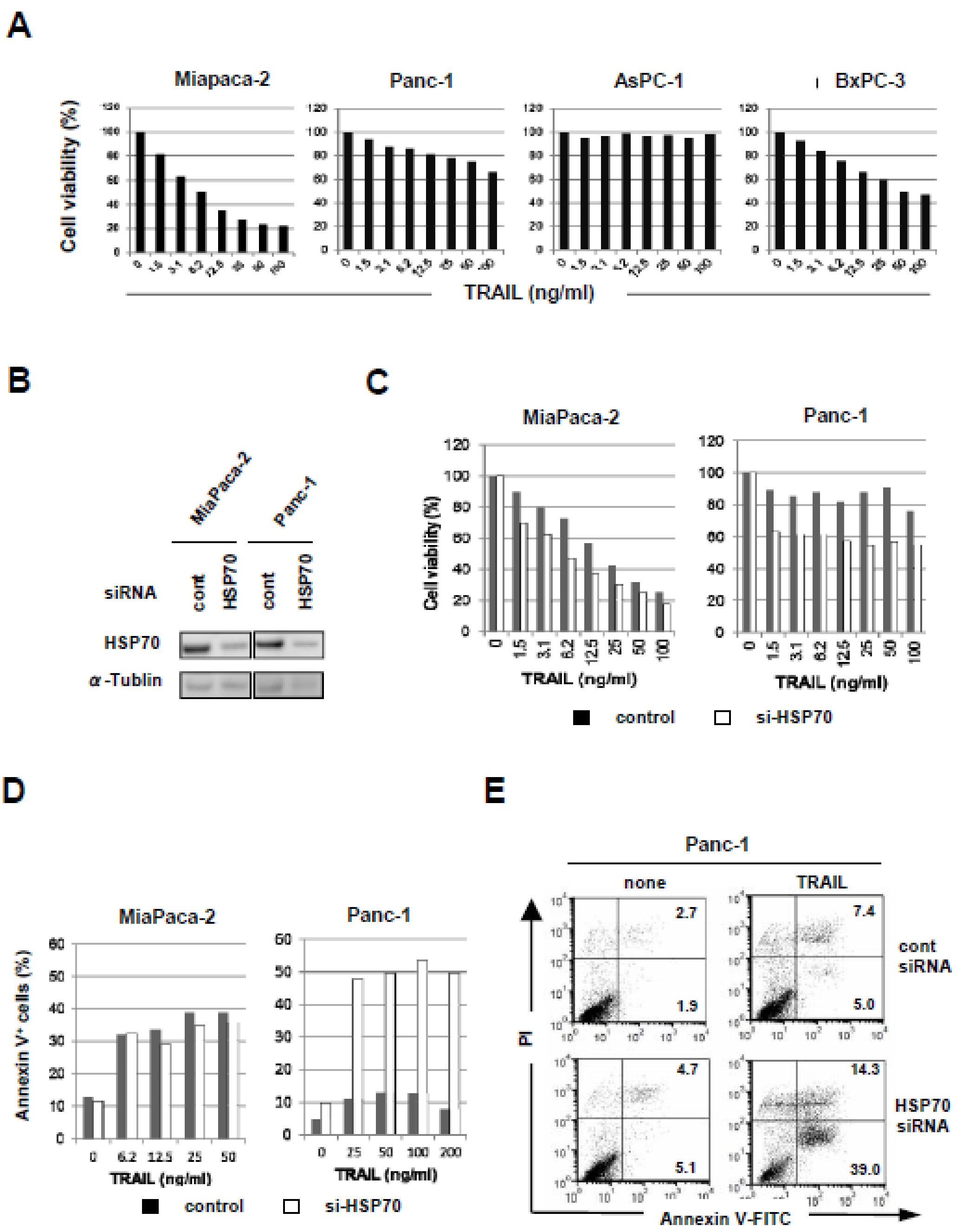

Figure 1. Protective role of HSP70 in TRAIL-induced antitumor effects. (**A**) Four cell lines were cultured with PFT-μ. After 48 h, cell viability (%) was determined by WST-8 assay. The data are the means of three wells. (**B**) After transfection of HSP70 siRNA or

control siRNA, HSP70 expression was evaluated by immunoblotting. α-Tubulin was used as the control. (**C** and **D**) MiaPaca-2 and Panc-1 cells which had been transfected with HSP70 siRNA or control siRNA 3 days previously were cultured with TRAIL. After 48 h, cell viability (%) and the percentage of Annexin V^+ cells were determined by WST-8 assay. and flow cytometry, respectively. The data are the means of three wells. (**E**) Representative flow cytometry result of Panc-1. The numbers represent the percentage of each subset.

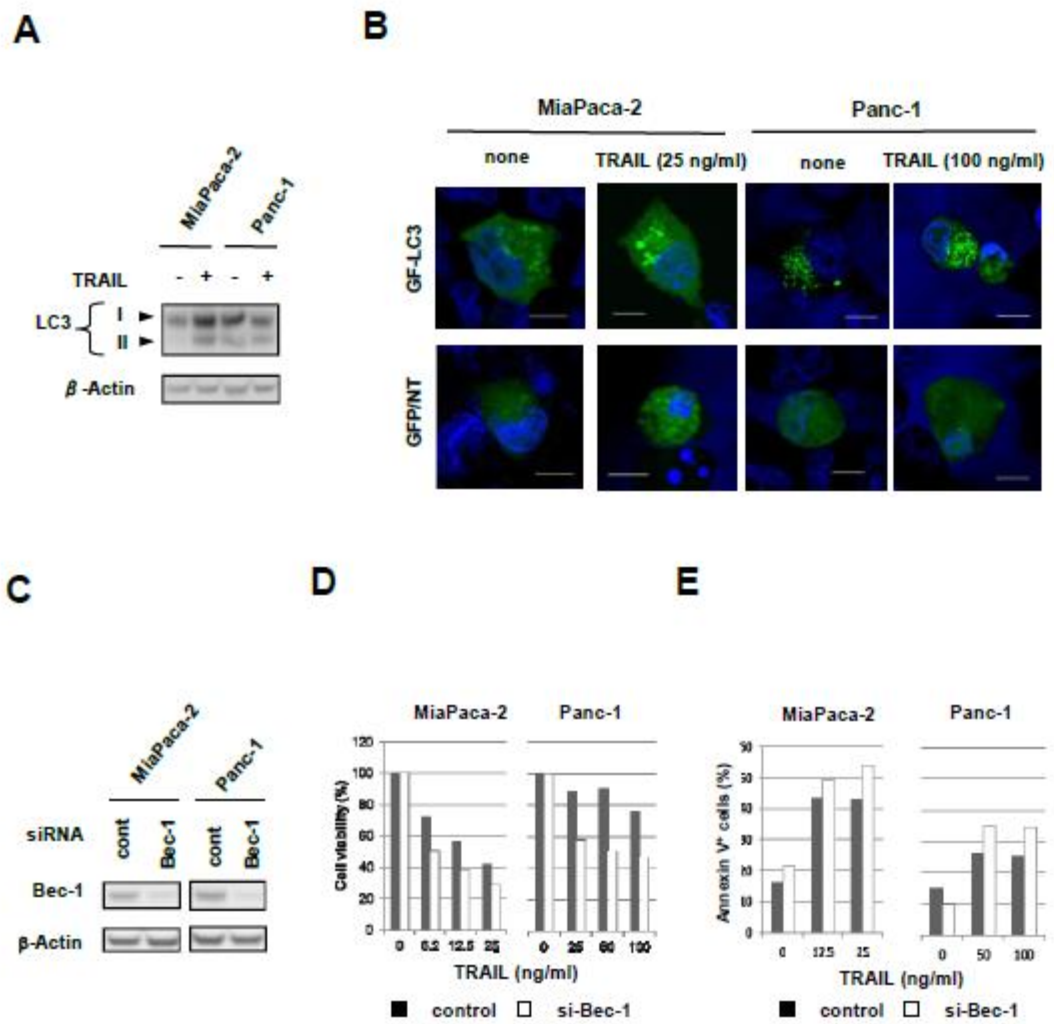

Figure 2. Protective role of autophagy in the antitumor effect of TRAIL on human pancreatic cancer cell lines. (**A**) MiaPaca-2 and Panc-1 cells were cultured with TRAIL at 25 and 100 ng/ml, respectively, for 24 h, and LC3 expression was evaluated by immunoblotting. β-Actin was used as the control. (**B**) MiaPaca-2 and Panc-1 cells that had been pre-transfected with a plasmid encoding GFP-LC3 or GFP/NT were cultured with the indicated TRAIL concentration. After 18 h, the expression of LC3 (green) and nuclear staining with Hoechst 33342 (blue) were visualized by confocal microscopy. Scale bar, 10 µm. (**C**) MiaPaca-2 and Panc-1 cells that had been transfected with Beclin-1 siRNA or control siRNA 3 days previously were evaluated by immunoblotting. β-Actin was used as the control. Bec-1, Beclin-1. (**D**) MiaPaca-2 and Panc-1 cells that had been transfected with Beclin-1 siRNA or control siRNA 3 days previously were cultured with TRAIL and their viabilities assessed by WST-8 assay. The data are the means of three wells. (**E**) Flow cytometry analysis was performed after staining with FITC-conjugated Annexin V and PI. The data are the means of three wells.

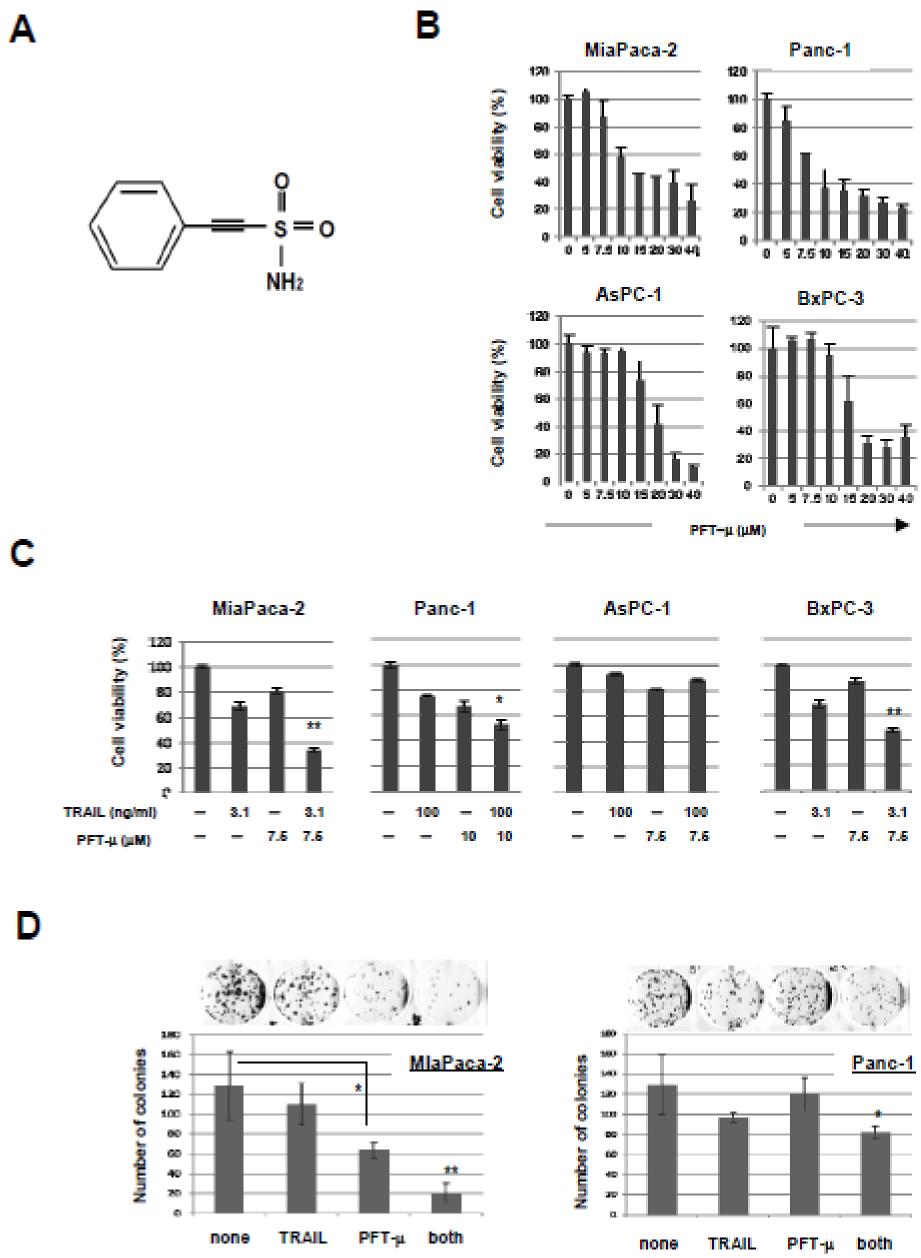

Figure 3. PFT-μ enhances the antitumor effects of TRAIL on pancreatic cancer cell lines. (**A**) The structure of PFT-μ. (**B**) Four pancreatic cancer cell lines were cultured with the indicated PFT-μ concentrations, and cell viability (%) was determined by the WST-8 assay after 48 h. Results are means ± SD of three wells. (**C**) Four cancer cell lines were

cultured with TRAIL and/or PFT-μ. After 48 h, cell viability (%) was determined by WST-8 assay. *P<0.05, **P<0.01 compared to the other three groups. (**D**) MiaPaca-2 and Panc-1 cells were cultured with TRAIL and/or PFT-μ for 2 days and without TRAIL and PFT-μ for an additional 10 days. The results are means ± SD of three wells. *P<0.05, **P<0.01.

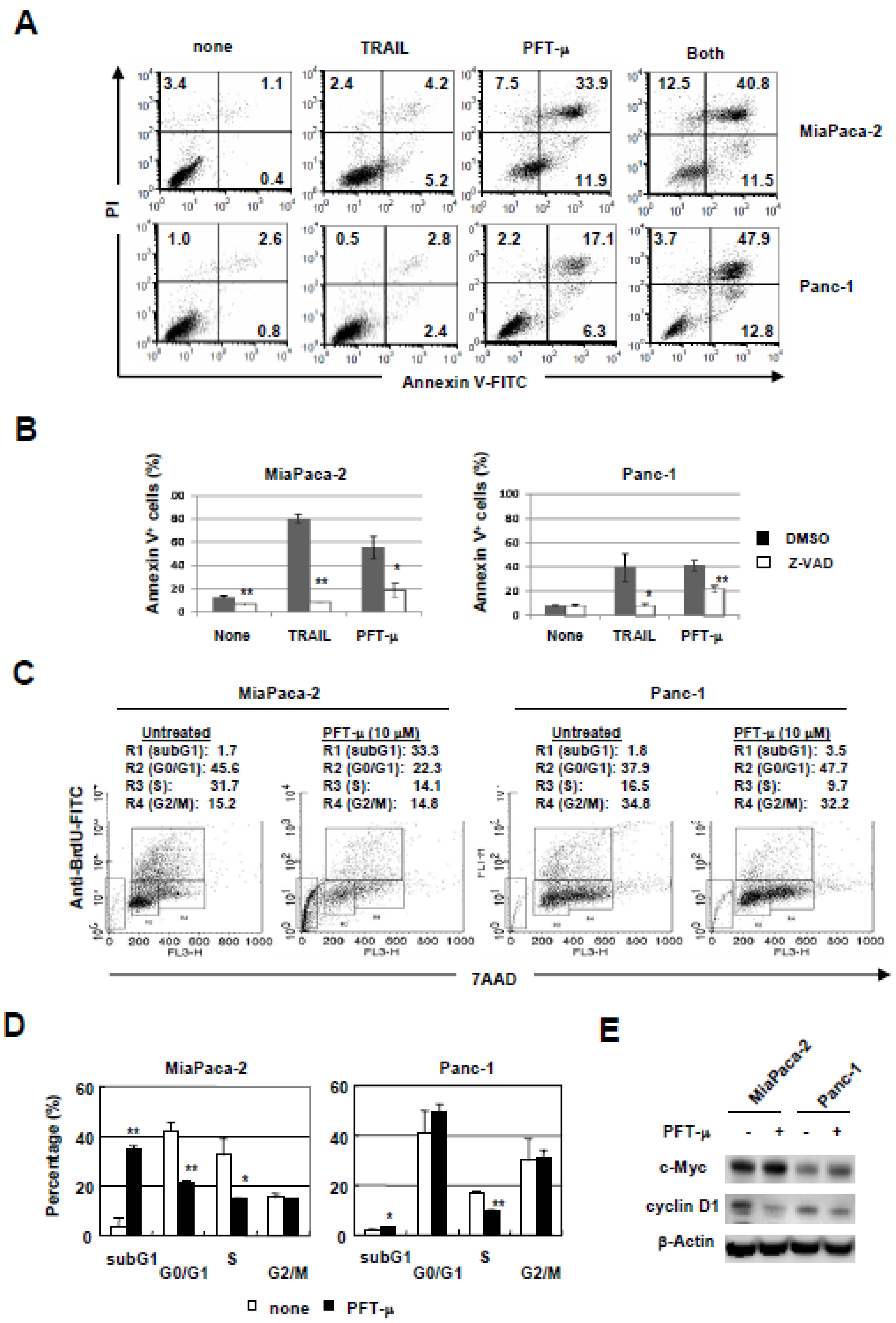

Figure 4. Induction of cell death and growth arrest in pancreatic cancer cells by PFT-μ.

(**A**) MiaPaca-2 and Panc-1 cells were cultured with TRAIL (20 and 100 ng/ml, respectively) and PFT-μ (15 μM). After 48 h, flow cytometry analysis was performed after staining with FITC-conjugated Annexin V and PI. A representative result is shown. The numbers represent the percentages of each subset. (**B**) MiaPaca-2 and Panc-1 cells were cultured with TRAIL (20 and 100 ng/ml, respectively) and PFT-μ (15 μM) in the presence of z-VAD or DMSO. After 48 h, flow cytometry analysis was performed after staining with FITC-conjugated Annexin V and PI. The results are shown as the means ± SD of three wells. **P*<0.05, ***P*<0.01. (**C**) MiaPaca-2 and Panc-1 cells were cultured with PFT-μ (10 μM) for 2 days. During the last 90 min for MiaPaca-2 cells and 5 h for Panc-1 cells, cells were cultured with BrdU (10 μM). Harvested cells were stained with 7AAD, and flow cytometry analysis was performed. Numbers represent the percentages of each subset. (**D**) The results are shown as the means ± SD of three wells. **P*<0.05, ***P*<0.01. (**E**) MiaPaca-2 and Panc-1 cells were cultured with PFT-μ (10 μM) for 2 days, and c-Myc and cyclinD1 protein levels were determined by immunoblotting. β-Actin was used as the control.

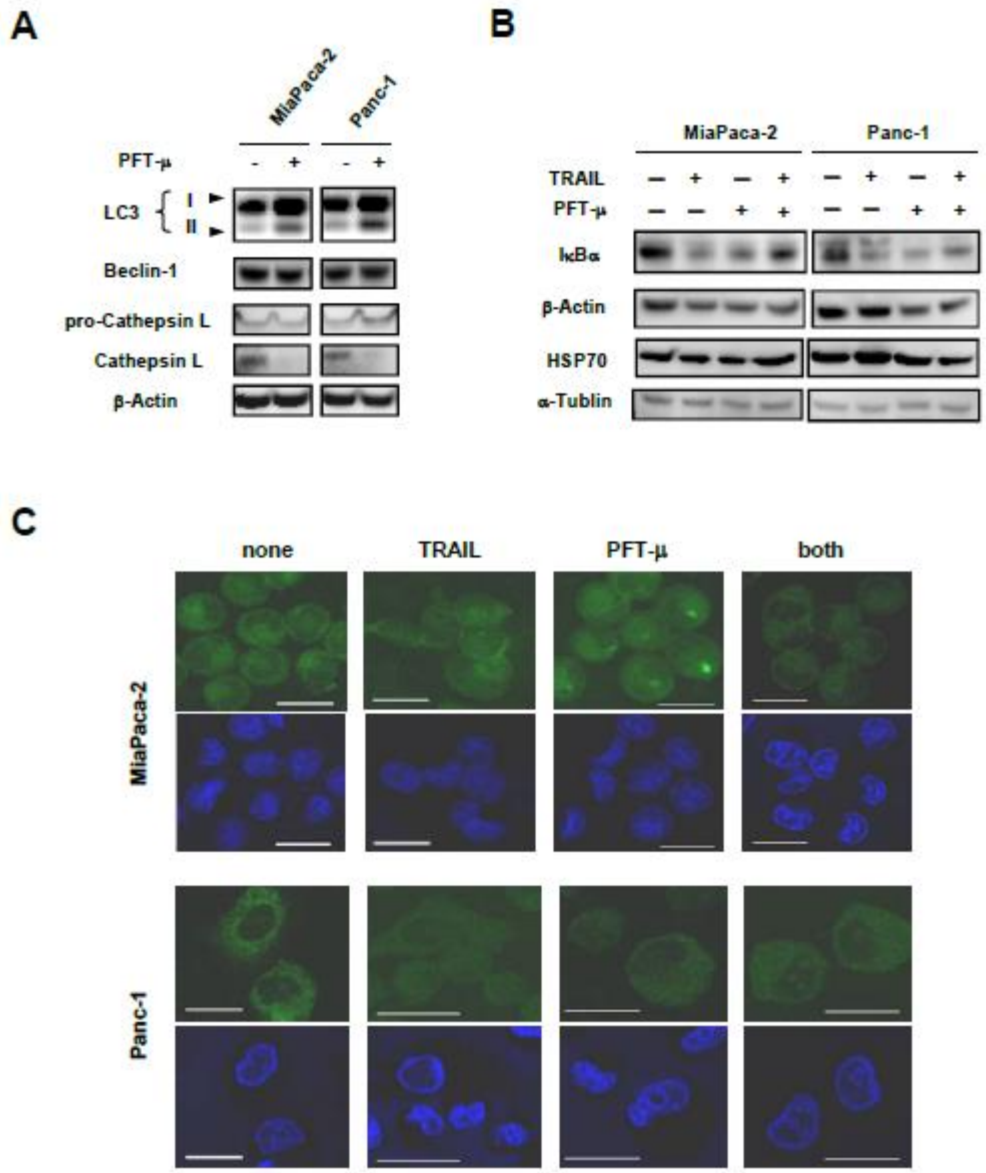

Figure 5. PFT-μ inhibits autophagy and TRAIL-associated NF-κB activation. (**A**) MiaPaca-2 and Panc-1 cells were cultured with PFT-μ (20 μM), and LC3, Beclin-1, pro-Cathepsin L, and Cathepsin L protein levels were determined by immunoblotting. β-Actin was used as the control. (**B**) After culture with PFT-μ (20 μM) for 5 h, MiaPaca-2

and Panc-1 cells were additionally cultured with TRAIL (25 ng and 100 ng/ml, respectively) for 30 min. Thereafter, IκBα and HSP70 expressions were determined by immunoblotting. β-Actin and α-tubulin were used as controls. (**C**) After culture with PFT-μ (20 μM) for 5 h, MiaPaca-2 and Panc-1 cells were additionally cultured with TRAIL (50 ng and 200 ng/ml, respectively) for 30 min. The expression of NF-κB p65 (green) and nuclear staining with Hoechst 33342 (blue) were visualized by confocal microscopy. Scale bar, 20 μm.

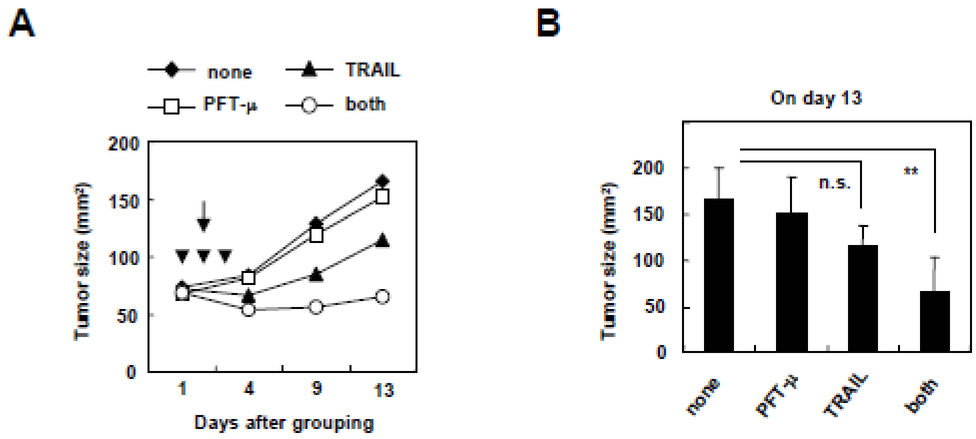

Figure 6. *In vivo* antitumor effects of TRAIL and PFT-μ in a xenograft mouse model.

(**A**) BALB *nu/nu* mice were subcutaneously inoculated in the right flank with MiaPaca-2 cells (4×10^6) with Matrigel (total volume, 200 μl). On day 20, the mice were pooled and divided into four groups. On days 1, 2, and 3 after grouping, the mice were intraperitoneally injected with PFT-μ (25 mg/kg; 100 μl). As a vehicle control, an identical volume of DMSO was injected. On day 2 after grouping, the mice were injected once intratumorally with 1.5 μg of TRAIL (50 μl). As a vehicle control, an identical volume of complete medium was injected. Thereafter, tumor size was measured twice weekly. Arrow heads (PFT-μ) and arrows (TRAIL) represent the day of treatment. Each group contained seven or eight mice. (**B**) Tumor size on day 13 after grouping. The results are shown as the means ± SD of seven or eight mice. *P < 0.05 (ANOVA with Scheffe's *post hoc* test). n.s., not significant.