

This remarkable tolerance to nutrient starvation is one of the key factors for survival and progression of pancreatic tumors. Therefore, agents that retard the tolerance of cancer cells to nutrient starvation represent a novel approach in anticancer drug discovery.¹⁵ Using this hypothesis, we established a novel antiausterity strategy for the discovery of anticancer agents that preferentially target tolerance to nutrient starvation by cancer cells. Previous work on this strategy has led to the discovery of a number of potent anticancer agents, such as arctigenin,¹⁵ angelmarin,¹⁶ kayeassamins A–I,^{17,18} and panduratinins,^{19,20} from the medicinal plants used in Japanese Kampo medicine and Southeast Asian countries.²¹ Interestingly, these compounds also strongly suppressed tumor growth in a xenograft model using pancreatic cancer cells.¹⁵ In our continued work, we recently found that a dichloromethane extract of the stem of *Uvaria dac* preferentially inhibited PANC-1 human pancreatic cancer cell survival under nutrient deprivation.²² Work-up of this bioactive extract led to the discovery of (+)-grandifloracin (GF) as a potent antiausterity agent that showed preferential toxicity to PANC-1 cells with a PC_{50} value of 14.5 μ M. In this study, we explored the underlying mechanism of GF-induced modulation of key regulatory proteins involved in tolerance to nutrient starvation in PANC-1 cells.

Materials and methods

Reagents

GF (Figure 1) was isolated from the stems of *U. dac* as described previously.²² GF purity was determined to be 95% by high-performance liquid chromatography. Conventional anticancer agents, ie, gemcitabine, 5-fluorouracil, 2-deoxyglucose, paclitaxel, podophyllotoxin, and camptothecin, were purchased from Sigma-Aldrich (St Louis, MO, USA). Each reagent was dissolved in dimethyl sulfoxide as a 10 mM stock solution and stored at -30°C until use. Dilution to give the desired concentration was performed prior to treatment. Dulbecco's phosphate-buffered saline was purchased from Nissui Pharmaceutical (Tokyo, Japan). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Wako Pure Chemical (Osaka, Japan). Sodium bicarbonate, potassium

chloride, magnesium sulfate, sodium dihydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, and phenol red were purchased from Wako Pure Chemical. HEPES was purchased from Dojindo Laboratories (Kumamoto, Japan). Fetal bovine serum was purchased from Nichirei Biosciences Inc. (Tokyo, Japan). Antibiotic/antimycotic solution was purchased from Sigma-Aldrich. The WST-8 cell counting kit was purchased from Dojindo Laboratories. Cell culture flasks and 96-well plates were obtained from Falcon Becton Dickinson Labware (BD Biosciences, San Jose, CA, USA). Nutrient-deprived medium was prepared according to a previously described protocol.¹⁴ Rabbit polyclonal antibodies to Akt, phosphoryl Akt (Ser473), mammalian target of rapamycin (mTOR), phosphoryl mTOR (Ser2448), Bcl-2, caspase 3, and LC3A/B were purchased from Cell Signaling Technology (Danvers, MA, USA). A goat polyclonal antibody to actin was purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). Horseradish peroxidase-conjugated goat polyclonal anti-rabbit and rabbit polyclonal anti-goat immunoglobulins were purchased from DakoCytomation (Glostrup, Denmark).

Cell line

The PANC-1 (RBRC-RCB2095) cell line was purchased from the Riken BRC Cell Bank (Ibaraki, Japan) and maintained in standard DMEM with 10% fetal bovine serum supplement, 100 U/mL of penicillin G, 0.1 mg/mL of streptomycin, and 0.25 μ g/mL of amphotericin B.

Preferential cytotoxic activity

The in vitro preferential cytotoxicity of GF was determined using a previously described procedure with a slight modification.²² In brief, human pancreatic cancer cells were seeded in 96-well plates (1.5×10^4 /well) and incubated in fresh DMEM at 37°C under humidified 5% CO_2 and 95% air for 24 hours. After the cells were washed with Dulbecco's phosphate-buffered saline, the medium was changed to serially diluted test samples in DMEM or nutrient-deprived medium, with the control and blank in each plate. After 24 hours of incubation, 100 μ L of DMEM containing 10% WST-8 cell counting kit solution was directly added to each well. After 3 hours of incubation, absorbance at 450 nm was measured (EnSpire[®] Multilabel Reader, PerkinElmer, Waltham, MA, USA). Cell viability was calculated from the mean values for three wells using the following equation:

$$\text{Cell viability (\%)} = \frac{[(\text{Abs}_{(\text{test sample})} - \text{Abs}_{(\text{blank})}) / (\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{blank})})] \times 100}$$

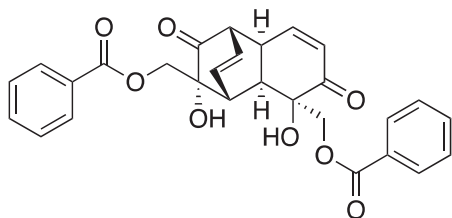


Figure 1 Chemical structure of (+)-grandifloracin.

Morphologic assessment

Cells were seeded in 60 mm dishes (1×10^6 cells) and incubated in a humidified CO₂ incubator for 24 hours to allow cell attachment. The cells were then washed twice with Dulbecco's phosphate-buffered saline and treated with 25 μ M GF in DMEM, nutrient-deprived medium, and the control. After 12 and 24 hours of incubation, the cells were treated with fluorescein-labeled annexin V and propidium iodide, and cell morphology was observed using an inverted Nikon Eclipse TS 100 microscope (40 \times objective) with phase-contrast and fluorescence modes. Microscopic images were taken using a Nikon DS-L-2 camera directly attached to the microscope.

Annexin V/dead cell assay

The annexin V/dead cell assay was performed in a Muse™ cell analyzer (Merck Millipore, Billerica, MA, USA) utilizing a Muse annexin V and dead cell kit. The assay utilizes phycoerythrin-labeled annexin V to detect phosphatidylserine on the external membrane of apoptotic cells. The kit contains the DNA dye, 7-aminoactinomycin D (7-AAD) for the exclusion of nonviable cells. Four populations of cells can be distinguished in this assay: nonapoptotic cells, annexin V (–) and 7-AAD (–); early apoptotic cells, annexin V (+) and 7-AAD (–); late-stage apoptotic and dead cells, annexin V (+) and 7-AAD (+); and necrotic nuclear debris, annexin V (–) and 7-AAD (+). The assay was performed according to the manufacturer's protocol. In brief, the cells were seeded in 60 mm dishes (1×10^6 cells) and incubated in a humidified CO₂ incubator for 24 hours to allow cell attachment. The cells were then washed twice with Dulbecco's phosphate-buffered saline and treated with 12.5 μ M GF, 25 μ M GF, or the control of nutrient-deprived medium for the indicated time periods. The cells were then harvested from the dish with trypsin to give single cell suspensions. Finally, 100 μ L of annexin V/dead reagent and 100 μ L of a single cell suspension were mixed in a microtube and incubated for 20 minutes at room temperature in the dark. The cells were then analyzed using the Muse cell analyzer, and 5,000 cell events were collected for each sample. The images were acquired as the screenshots of the processed data and the text size was edited for clarity.

Western blot analysis

Proteins were separated by gel electrophoresis on a polyacrylamide gel containing 0.1% sodium dodecyl sulfate and transferred to polyvinylidene fluoride membranes. The membranes were blocked with Block Ace® (DS Pharma Medical, Suita, Japan), washed with Dulbecco's phosphate-buffered saline

containing 0.1% polyoxyethylene (20) sorbitan monolaurate (Wako Pure Chemical), and incubated overnight with primary antibodies diluted in Can Get Signal® (Toyobo, Osaka, Japan). After washing, the membranes were incubated for 45 minutes at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-goat immunoglobulins as the secondary antibody. The bands were detected with an enhanced chemiluminescence solution (PerkinElmer). The images were analyzed using Image Studio software version 3.1.4.

Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test. A *P*-value < 0.05 was considered to be statistically significant.

Results

GF showed preferential cytotoxicity in a concentration-dependent manner

The PANC-1 cell line is highly resistant to nutrient deprivation and shows an extraordinary ability to survive for >48 hours even under complete nutrient starvation. GF remarkably diminished tolerance to nutrient starvation in a concentration-dependent manner (Figure 2A). Cells exposed to GF at 25 μ M showed 100% cell death within 24 hours in nutrient-deprived medium, with a PC₅₀ value of 14.5 μ M; however, no toxicity was observed in nutrient-rich DMEM.

GF sensitized PANC-1 cell death under glucose/serum-deprived conditions

To determine the conditions under which GF induces sensitivity to nutrient starvation resulting in cell death, the PANC-1 cells were treated with 25 μ M GF under various nutrient conditions of glucose, amino acids, and serum. Cell viability was measured 24 hours after treatment. As shown in Figure 2B, GF was found to be toxic during glucose or serum deprivation, irrespective of the presence or absence of amino acids. In the presence of glucose and serum, cell viability was 100%. However, removal of serum led to a decrease in cell viability to 73% and 69% in the presence or absence of amino acids, respectively. Similarly, removal of glucose also led to a significant decrease in cell viability to 66%. Removal of both glucose and serum decreased cell viability to 2%.

Conventional anticancer agents are ineffective against PANC-1 cells in nutrient-deprived medium

The preferential cytotoxicity of GF was compared with that of several conventional anticancer agents, including

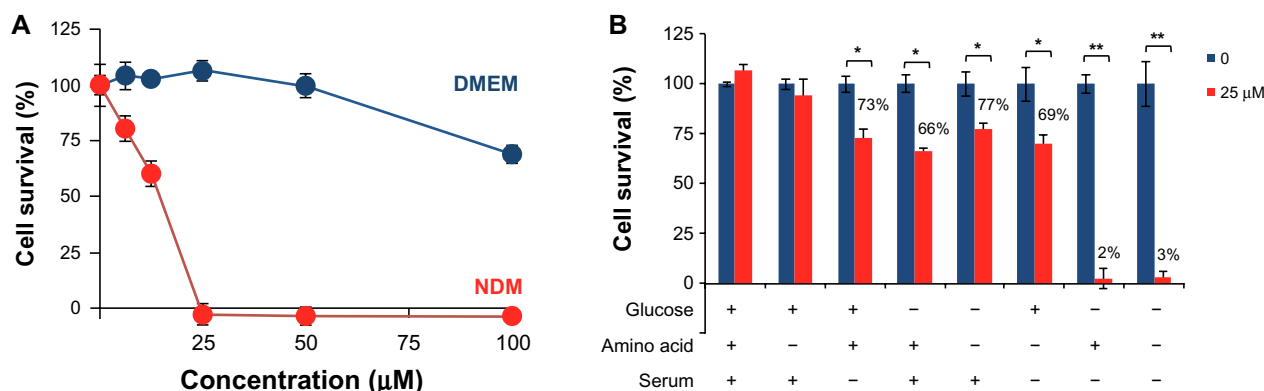


Figure 2 Effect of (+)-grandifloracin on PANC-1 cell survival after 24 hours in NDM and normal medium (DMEM). **(A)** Effect of (+)-grandifloracin concentration on cell survival in NDM and DMEM. **(B)** Effects of medium components, ie, glucose, amino acids, and serum. Data are expressed as the mean ± standard deviation, n=3. *P<0.05; **P<0.01 indicate significant difference from the control.

Abbreviations: NDM, nutrient-deprived medium; DMEM, Dulbecco's Modified Eagle's Medium.

gemcitabine, 5-fluorouracil, 2-deoxyglucose, paclitaxel, camptothecin, and podophyllotoxin, using PANC-1 cells grown in nutrient-deprived medium versus DMEM (Figure 3). All tested agents were virtually inactive in nutrient-deprived medium; however, paclitaxel and camptothecin showed weak activity in nutrient-rich DMEM at the maximum tested dose of 100 µM after 24 hours. Because some of the conventional anticancer agents showed weak activity in DMEM, their effects during prolonged treatment were also evaluated by monitoring their cytotoxicity after 24, 48, and 72 hours. As shown in Figure 4, gemcitabine and 5-fluorouracil weakly decreased cell viability 72 hours after treatment. However, these compounds did not show a clear concentration-dependent effect. 2-Deoxyglucose was completely inactive. Paclitaxel and podophyllotoxin were found to reduce cell viability after 72 hours, but the effect was not concentration-dependent. On the other hand, camptothecin

exhibited strong activity with cell viability of <25% at 10 µM 48 hours after treatment.

Assessment of GF-induced apoptosis

To investigate whether GF-induced cell death in nutrient-deprived medium involves apoptosis, the cell morphology was examined. As shown in Figure 5, at 25 µM, GF induced a marked change in PANC-1 cell morphology within 8 hours. However, the cells lacked the classical signs of apoptosis, such as shrinkage or fragmentation into membrane-bound apoptotic bodies. Instead, swelling and rupture of cell membranes and disruption of cellular organelles appeared to be closer to a necrotic-type cell death. Staining with annexin V/propidium iodide reagent showed an increased population of cells containing Annexin V (green fluorescence) and propidium iodide (red fluorescence). Annexin V is a Ca²⁺-dependent phospholipid-binding protein with

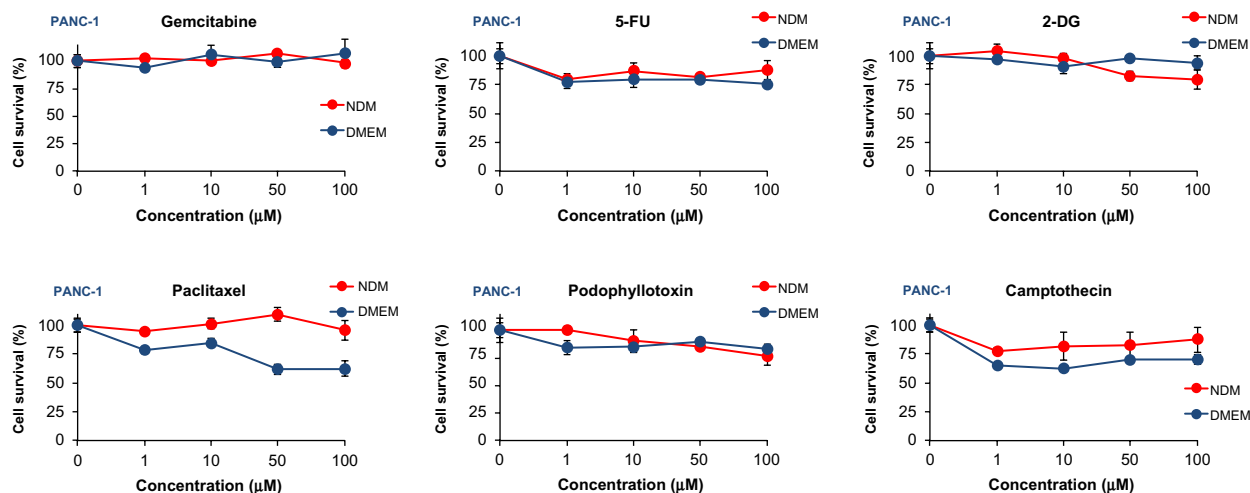


Figure 3 Effect of conventional anticancer agents against PANC-1 cells after 24 hours in NDM and DMEM. Data are expressed as the mean ± standard deviation, n=3. **Abbreviations:** NDM, nutrient-deprived medium; DMEM, Dulbecco's Modified Eagle's Medium; 5-FU, 5-fluorouracil; 2-DG, 2-deoxyglucose.

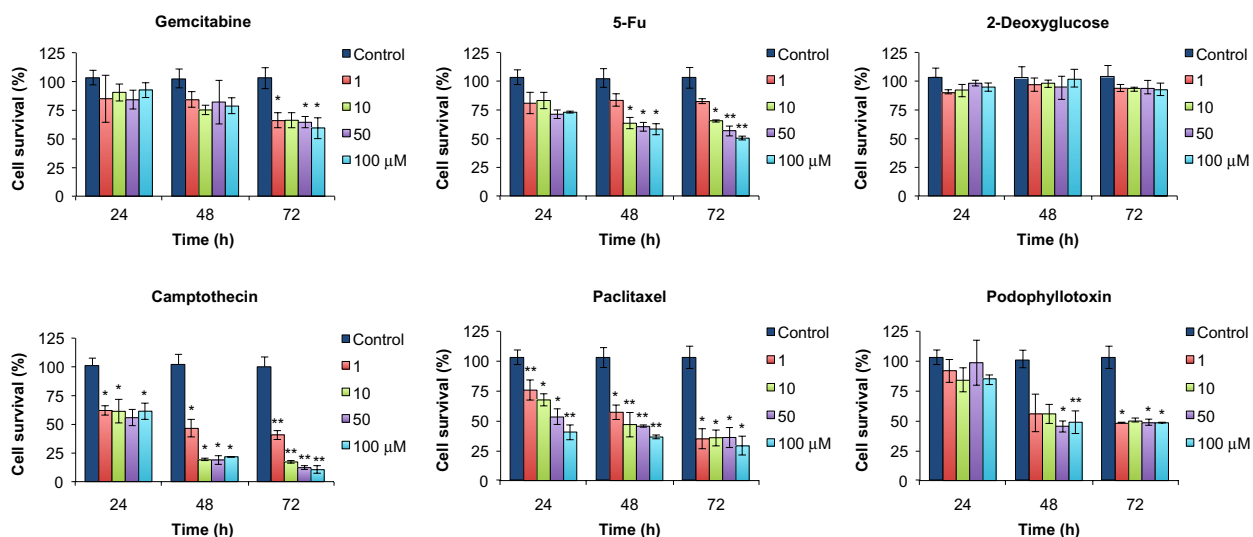


Figure 4 Assessment of cytotoxicity of conventional anticancer agents against PANC-1 cells in Dulbecco's Modified Eagle's Medium. Data are expressed as the mean \pm standard deviation, $n=3$. * $P<0.05$; ** $P<0.01$ indicates a significant difference from the control.

Abbreviation: 5-FU, 5-fluorouracil.

high affinity for phosphatidylserine. Translocation of phosphatidylserine to the external cell surface occurs both in apoptosis and necrosis. We further performed flow cytometric analysis of cells treated with GF utilizing the Muse Annexin V and dead cell kit, which contains 7-AAD as a dye for exclusion of nonviable cells. 7-AAD is impermeable to viable cells and does not stain viable or early apoptotic cells. In late apoptotic and necrotic cells, the integrity of the cell membrane decreases, which allows 7-AAD to pass through the membranes, intercalate into nucleic acids and DNA, and display red fluorescence. As shown in Figure 6,

the cells are predominantly stained with both Annexin V and 7-AAD within 12 hours in a concentration-dependent manner. In the control of nutrient-deprived medium, more than 90% of the cells survived. After treatment with GF, this cell population decreased markedly to 72% (12.5 μM) and 29% (25 μM), with an increase in the late apoptotic/necrotic cell population from 1% (control) to 15% (12.5 μM) and 61% (25 μM), respectively (Figure 6). We further performed Western blot analysis to examine GF-induced apoptosis. Treatment with GF neither led to cleavage of caspase-3 nor showed Bcl-2 inhibition (data not shown).

GF inhibits Akt/mTOR activation

Akt is a prosurvival factor that is activated in a majority of tumors and regulates cellular functions such as cell cycle progression, cell migration, invasion, and angiogenesis. High Akt activation has been associated with tolerance to nutrient starvation and survival in an austerity environment.¹⁴ Therefore, the effect of GF on Akt activation was investigated by Western blot analysis. As shown in Figure 6, Akt phosphorylation at Ser473 was completely inhibited by GF in a concentration-dependent as well as time-dependent manner in nutrient-deprived medium. GF also strongly suppressed total Akt. mTOR is a downstream effector of Akt and is frequently activated in various cancer types, where it is involved in tumor progression and metastasis.²³ Therefore, we tested whether GF has any modulatory activity against mTOR activation. As shown in Figure 7, addition of 25 μM GF completely inhibited mTOR phosphorylation at Ser2448 6 hours after treatment.

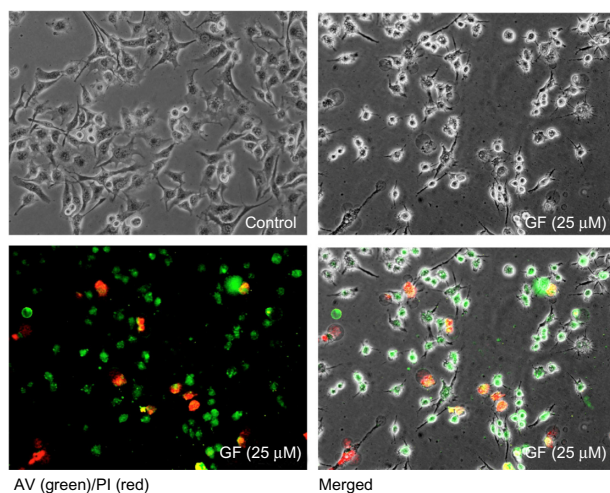


Figure 5 Effect of GF (25 μM) on PANC-1 cell morphology after 8 hours in NDM. Phase-contrast (upper left), fluorescent (lower left), and merged (lower right) images of PANC-1 cells.

Abbreviations: AV, Annexin V; PI, propidium iodide; NDM, nutrient-deprived medium; GF, (+)-grandifloracin.

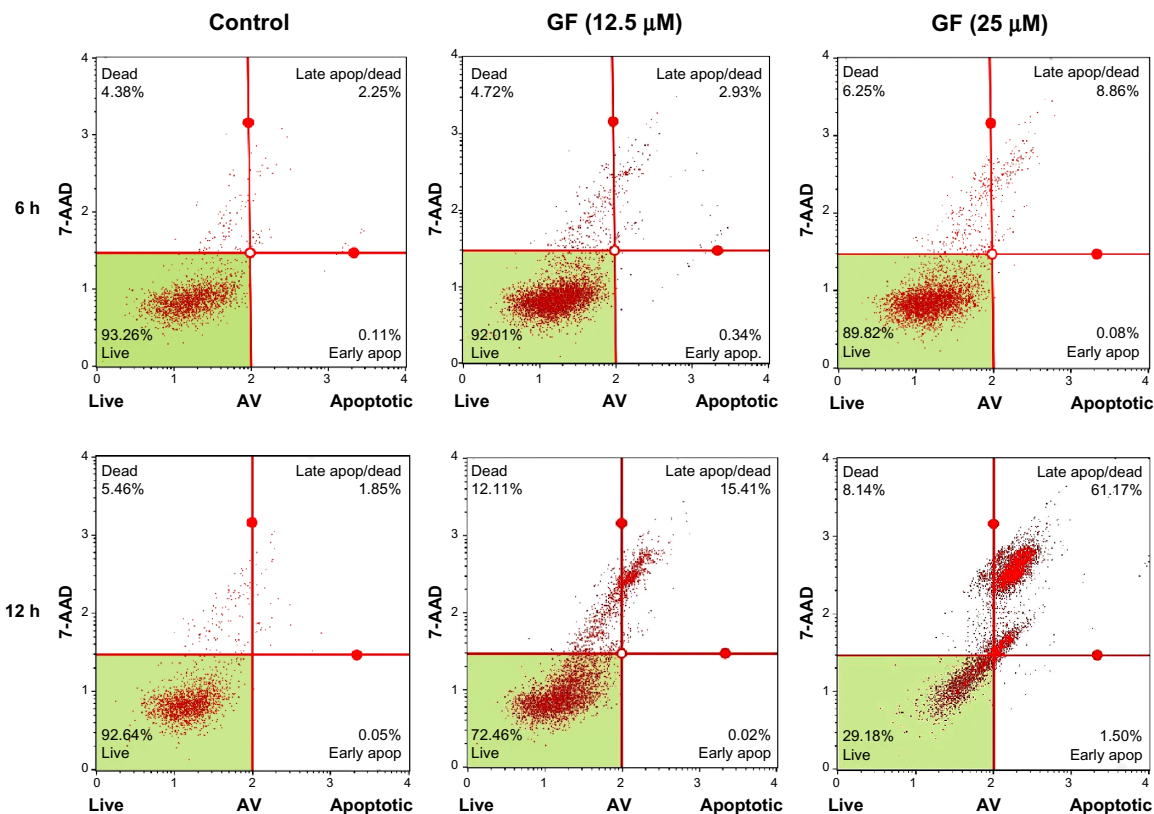


Figure 6 Assessment of apoptosis by GF. PANC-1 cells were treated with vehicle or GF (12.5 μM and 25 μM) in nutrient-deprived medium. After treatment (6 hours and 12 hours), the cells were treated with Annexin V/7-AAD reagent and cytometric analysis was performed.

Abbreviations: Apop, apoptotic; AV, Annexin V; GF, (+)-grandifloracin; 7-AAD; 7-aminoactinomycin D.

GF-induced autophagy in PANC-1 cells

Because no apoptotic cell death was observed in cells treated with GF, we speculated that GF might have induced autophagy. Therefore, expression of the autophagic marker microtubule-associated protein-light chain 3 (LC3), the cytoplasmic form

of LC3-I (16 kDa), and the preautophagosomal and autophagosomal membrane-bound form of LC3-II (14 kDa) was examined by Western blot. The PANC-1 cells were cultured for varying time periods at different GF concentrations. As shown in Figure 7, no apparent differences were observed in LC3-I

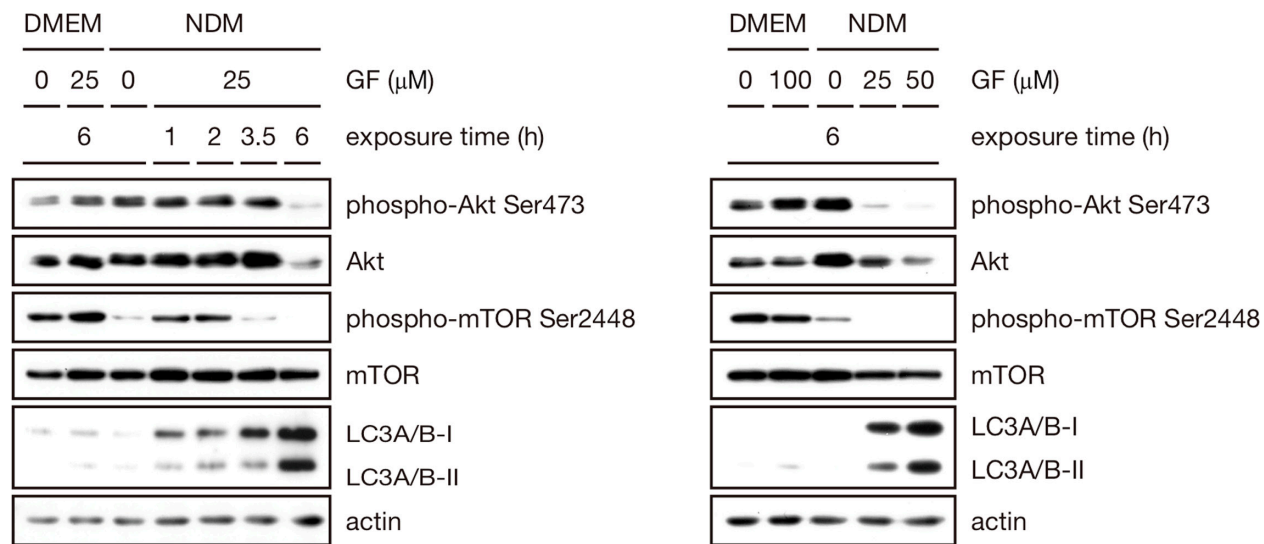


Figure 7 Effect of GF against Akt, mTOR, LC3A/B I, and LC3A/B II.

Abbreviations: GF, (+)-grandifloracin; NDM, nutrient-deprived medium; DMEM, Dulbecco's Modified Eagle's Medium.

and LC3-II expression in the controls of both DMEM and nutrient-deprived medium. However, treatment with GF led to an enhancement in the expression of both LC3-I and LC3-II in a concentration-dependent as well as time-dependent manner. In nutrient-deprived medium, treatment with 25 μM GF led to incremental increases of eight-fold, 13-fold, and 22-fold in LC3-I expression with respect to the control after 2, 3.5, and 6 hours, respectively. Similarly, increases of 141-fold, 146-fold, and 659-fold in LC3-II expression were observed with respect to the control after 2, 3.5, and 6 hours, respectively.

Discussion

Pancreatic cancer is associated with the lowest 5-year survival rate of any known cancer and is largely resistant to conventional chemotherapeutic agents. Although the median survival rate of the disease is only 6 months, some recent progress has been reported with FOLFIRINOX (folinic acid + 5-fluorouracil + irinotecan + oxaliplatin) and erlotinib.^{24,25} However, new alternatives are urgently needed to improve the clinical outcome for patients diagnosed with pancreatic cancer. Pancreatic tumors are hypovascular and supply only a limited amount of essential nutrients and oxygen to aggressively proliferating cells. Consequently, these cells live in a hostile microenvironment under chronic metabolic stress conditions. For survival, these cells activate adaptive mechanisms such as autophagy.^{26,27}

Autophagy is a homeostatic and evolutionarily conserved cellular pathway whereby cellular proteins and organelles are engulfed by autophagosomes, digested in lysosomes, and recycled in order to sustain cellular metabolism.²⁸ The process is activated in response to nutrient and energy starvation and acts as a survival mechanism to cope with diverse stresses in the tumor microenvironment.²⁸ Autophagy has been reported to be activated in colorectal cancer cells and to contribute to the tolerance to nutrient deprivation.²⁹ However, in the clinical setting, autophagy has been reported to serve as an alternative mechanism of programmed cell death that leads to tumor suppression.³⁰ One of the notable examples of a proautophagic cytotoxic drug that has demonstrated therapeutic benefits in several apoptosis-resistant cancer types in a clinical trial is temozolomide.³⁰ Several mechanisms have been suggested to explain the role of autophagy in suppression of tumorigenesis. Maintenance of genomic stability by clearance of damaged mitochondria and protein aggregates is considered one of the major mechanisms of tumor suppression by autophagy.³¹ Further, excessive metabolic stresses in the tumor microenvironment often lead to necrotic cell death. Activation of autophagy under such circumstances prevents necrotic cell

death and suppresses inflammation, which is known to increase tumor growth. Because the therapeutic goal of cancer treatment has been to trigger tumor-selective cell death, accelerating autophagy in apoptosis-resistant cancer cells would be an attractive alternative strategy in cancer therapy.

In the present study, GF does not appear to induce apoptosis but rather to operate by an alternative mechanism of programmed cell death, ie, autophagy. A marked activation of the autophagy marker LC3-II was observed after treatment with GF in a concentration-dependent and time-dependent manner. This was observed not only under nutrient-deprived conditions but also under nutrient-rich conditions, suggesting that GF is indeed an activator of autophagy. However, the effect of GF in nutrient-deprived medium was found to be highly significant compared with that in the control of nutrient-deprived medium at concentrations of 25 μM and 50 μM within 6 hours. Although a basal level of LC3-II protein is observed in the control of nutrient-deprived medium, it is activated within one hour after treatment with GF, which was found to be hyperactivated with respect to time as shown in Figure 5. This suggests that GF-induced autophagy mediates the death of PANC-1 cells preferentially during nutrient starvation.

The serine/threonine kinase Akt/mTOR pathway is constitutively activated in a majority of human pancreatic cancer cell lines. Activation of this pathway has been attributed to the survival of cancer cells in the heterogeneous tumor microenvironment, which confers resistance to chemotherapy and radiotherapy.¹⁴ Akt has been found to be overexpressed in pancreatic cancer cells during extreme nutrient deprivation. Increased Akt expression is one of the austerity markers that enables tumor cells to survive and proliferate in the hostile hypovascular tumor microenvironment.¹⁴ Therefore, inhibition of the Akt pathway might have therapeutic value in cancer patients. A number of antiausterity agents such as arctigenin, kigamicin D, and pyrvinium pamoate have been found to strongly suppress Akt activation, which suggests that inhibition of Akt phosphorylation by these compounds is partially responsible for the preferential cytotoxicity observed under nutrient deprivation.^{15,32,33} However, the manner in which Akt inhibition affects downstream signaling under austerity conditions remains largely unknown. In the present study, GF suppressed both total Akt and phospho(Ser473) Akt in a time-dependent as well as concentration-dependent manner. It has been reported that mTOR is frequently inappropriately activated in many cancer types, and development of drugs that inhibit mTOR is an alluring therapeutic target in cancer therapy. mTOR is a downstream effector of the PI3K/AKT pathway and is composed of two distinct complexes,

ie, mTORC1 and mTORC2. In the present study, although the effects of GF on each multiprotein complex were not elucidated, complete inhibition of mTOR phosphorylation at Ser2448 was observed. mTOR inhibitors, such as temsirolimus and everolimus, have been approved by the US Food and Drug Administration for the treatment of renal cell carcinoma, primitive neuroectodermal tumor, and giant cell astrocytoma.³⁴ In this regard, GF is a dual inhibitor of the principal survival factors, Akt and mTOR, in tumors. Because pancreatic tumors are highly resistant to current chemotherapeutic agents that induce apoptosis, induction of an alternative cell death mechanism exhibited by GF represents a novel attractive candidate for preclinical evaluation.

Acknowledgment

This work was supported by a grant from the Toyama Support Center for Young Principal Investigators in Advanced Life Sciences, Japan, and a Grant in Aid for Scientific Research (No 24510314) from the Japan Society for the Promotion of Science.

Disclosure

The authors report no conflict of interest in this work.

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