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Mitochondrial inhibitors show preferential cytotoxicity to human pancreatic cancer PANC-1 cells under glucose-deprived conditions

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ABSTRACT

Large areas of tumor are nutrient-starved and hypoxic due to a disorganized vascular system. Therefore, we screened small molecules to identify cytotoxic agents that function preferentially in nutrient-starved conditions. We found that efrapeptin F had preferential cytotoxicity to nutrient-deprived cells compared with nutrient-sufficient cells. Because efrapeptin F acts as a mitochondrial complex V inhibitor, we examined whether inhibitors of complex I, II, III, and V function as cytotoxic agents preferentially in nutrient-deprived cells. Interestingly, these inhibitors showed preferential cytotoxicity to nutrient-deprived cells and caused cell death under glucose-limiting conditions, irrespective of the presence or absence of amino acids and/or serum. In addition, these inhibitors were preferentially cytotoxic to nutrient-deprived cells even under hypoxic conditions. Further, efrapeptin F showed antitumor activity *in vivo*. These data indicate that mitochondrial inhibitors show preferential cytotoxicity to cancer cells under glucose-limiting conditions, and these inhibitors offer a promising strategy for anticancer therapeutic..

Introduction

Solid tumors have large areas starved for nutrients and oxygen that arise from immature and irregular distribution of blood vessels [1,2]. In particular, hypovascular tumors such as pancreatic cancers show an inherent ability to tolerate such severe growth conditions. Certain human pancreatic cancer cell lines, including PANC-1, AsPC-1, BxPC-3 and KP-3, exhibit marked environmental tolerance and can survive for prolonged periods of time in nutrient-deprived conditions [3]. Tolerance of these cancer cells to nutrient starvation has been associated with the activity of protein kinase B (PKB)/Akt. The PI3K-AKT-TOR signaling promotes cell proliferation and inhibits apoptosis. In addition, activation of Akt has been reported to stimulate cell survival, transformation, metastasis and angiogenesis [4,5]. Kigamicin D, a novel compound discovered from the culture broth of *Amycolatopsis* sp. ML630-mF1, blocks activation of Akt and exhibits preferential cytotoxicity to cancer cells under nutrient-deprived conditions compared to nutrient-rich conditions [6–8]. AG1024 and I-OMe-AG538, specific inhibitors of insulin-like growth factor-1 receptor tyrosine kinase, are also found to be cytotoxic to nutrient-deprived cells [9]. Therefore, agents active in nutrient-deprived conditions could function as anticancer agents.

Energy production is important for cell survival. The metabolism within a solid tumor is markedly different from that of the surrounding normal tissue [10–13]. Increased aerobic glycolysis is uniquely observed in cancers, thereby cancer cells use elevated amounts of glucose as a carbon source for anabolic reactions. However, part of the tumor is in a state of nutrient depletion. Tumor cells respond to nutrient-deprived conditions and adapt their metabolism to obtain amino acids. Autophagy is a catabolic process by which cells supply amino acids from self-digested organelles; cancer cells are likely to use autophagy to obtain amino acids as alternative energy sources [14]. Thus, their metabolic shift to the tumor microenvironment could represent a possible target for antitumor therapy. In this study, we screened natural products such as microbial metabolites to identify agents that preferentially reduce the survival of nutrient-deprived cancer cells. The screen identified efrapeptin F, which is produced by fungi and functions as a cytotoxic agent preferentially against human pancreatic cancer cells in glucose-limiting conditions.

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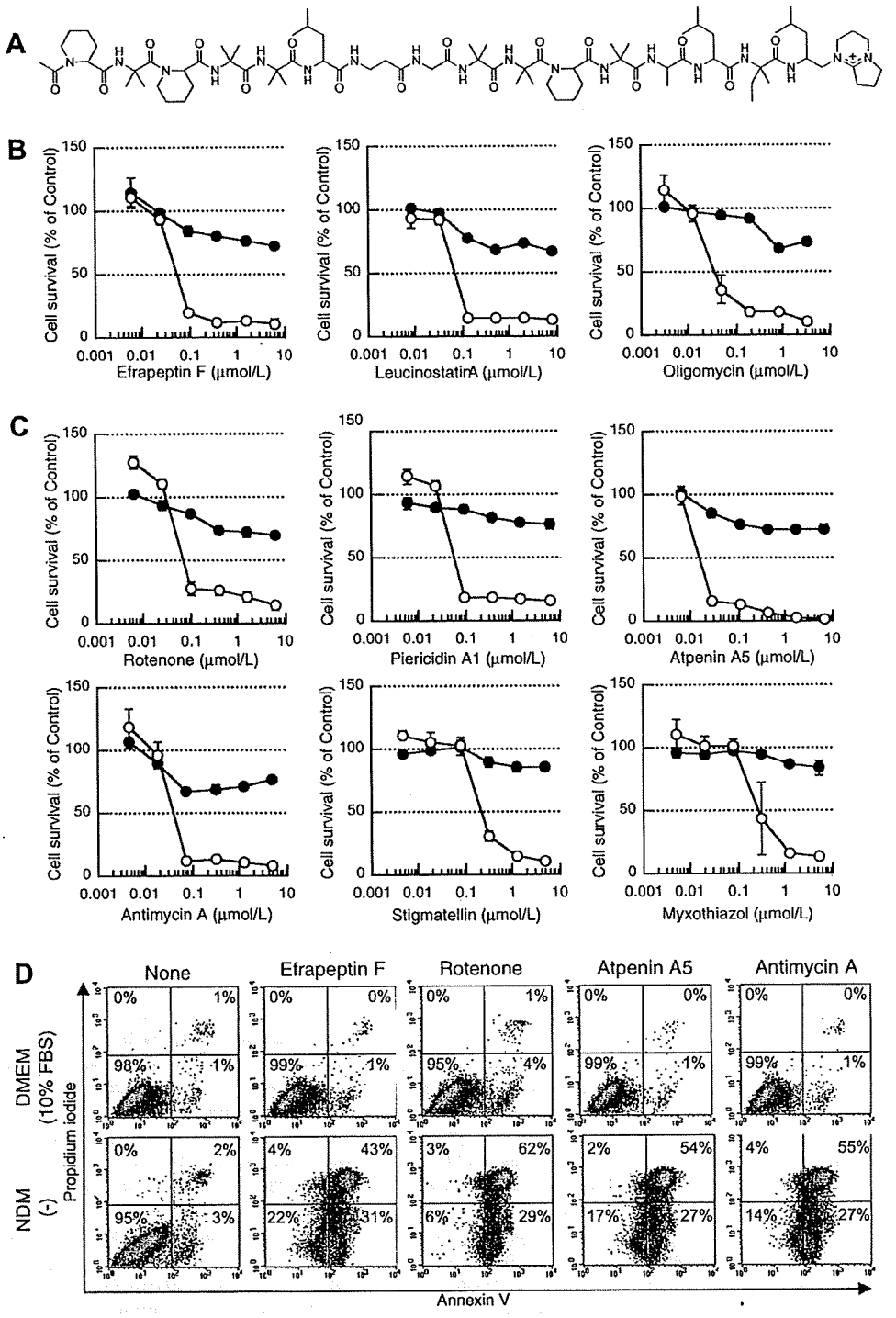
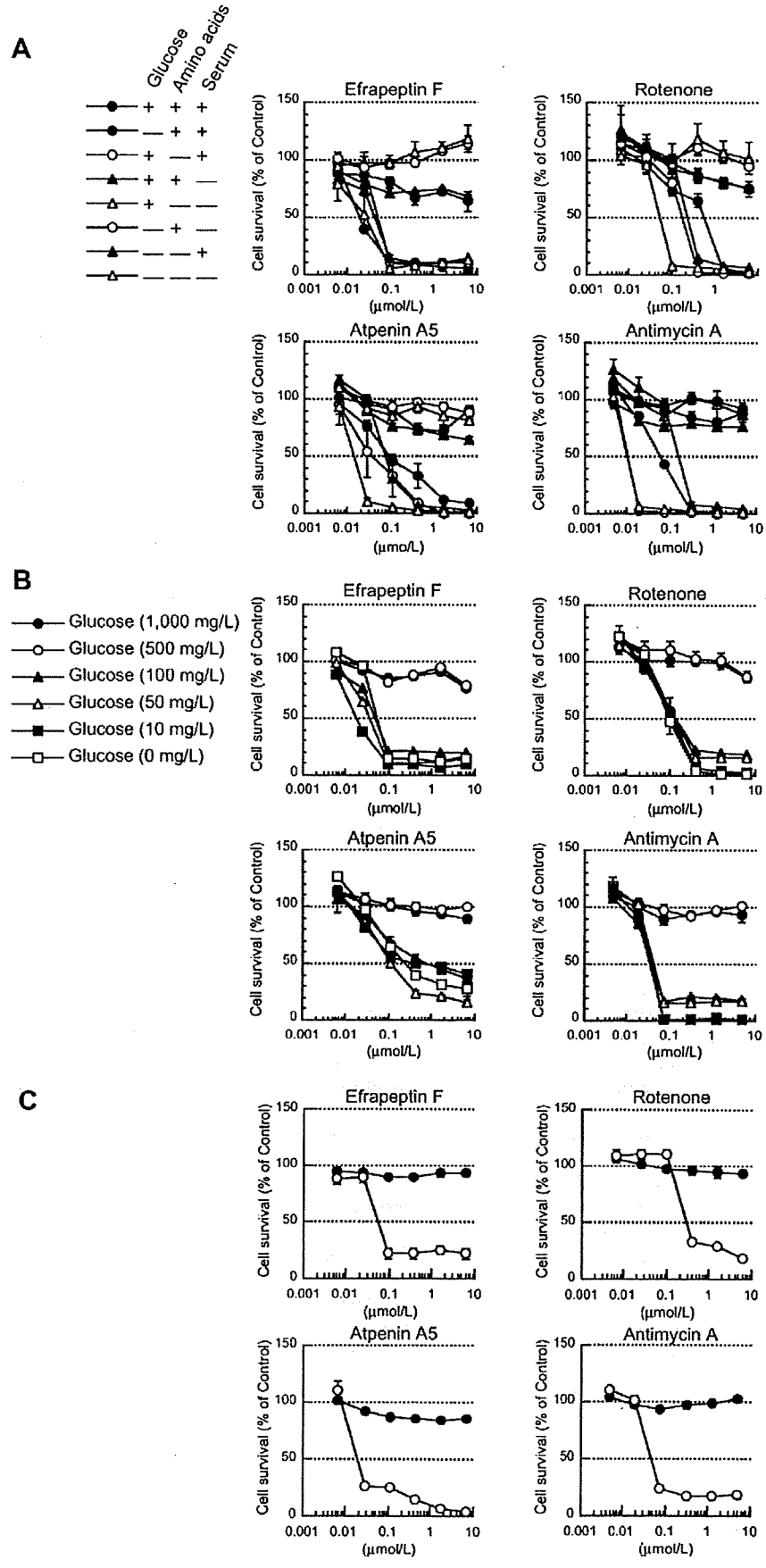


Fig. 1. Effect of efrapeptin F and mitochondrial inhibitors on PANC-1 survival under nutrient-deprived conditions. (A) Structure of efrapeptin F. (B) Effect of efrapeptin F and complex V inhibitors (leucinostatin A and oligomycin) on survival of PANC-1 cells in normal medium, DMEM (10% FBS) (●) and nutrient-deprived medium, NDM (−) (○). PANC-1 cells were incubated in DMEM (10% FBS) for 24 h. The cells were then washed with PBS and the medium was replaced with either fresh DMEM (10% FBS) or NDM (−). The indicated concentrations of efrapeptin F and complex V inhibitors were added to each well and the cells were incubated for 24 h. Cell viability was determined using the MTT assay. (C) Effect of complex I, II and III inhibitors on survival of PANC-1 cells in DMEM (10% FBS) (●) and NDM (−) (○). Rotenone and Piericidin A_1 were used as complex I inhibitors. Atpenin A_5 was as complex II inhibitors. Antimycin A, myxothiazol and stigmatellin were as complex III inhibitors. PANC-1 cells were incubated with inhibitors in DMEM (10% FBS) or NDM (−) for 24 h. (D) Flow cytometric analysis of PANC-1 cells treated with each inhibitor. PANC-1 cells were incubated with 0.1 μmol/L of mitochondrial inhibitors in DMEM (10% FBS) or NDM (−) for 24 h. The cells were stained with annexin V-FITC and propidium iodide and then analyzed using a flow cytometer.


A
Glucose
Amino acids
Serum
Efrapeptin F
Rotenone
Atpenin A5
Antimycin A
Cell survival (% of Control)
(μmol/L)
B
Glucose (1,000 mg/L)
Glucose (500 mg/L)
Glucose (100 mg/L)
Glucose (50 mg/L)
Glucose (10 mg/L)
Glucose (0 mg/L)
C

Materials and methods

Inhibitors. Efrapeptin F and Atpenin A_5 were purified from microbial culture extracts supplied by Meiji Seika Kaisha in our laboratory [15–18]. Rotenone and antimycin A were obtained from Sigma–Aldrich (St. Louis, MO).

Cell lines and culture conditions. Human pancreatic cancer PANC-1 cells and prostate cancer PC-3 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA), 100,000 U/L penicillin G, and 100 mg/L streptomycin. Nutrient starvation was achieved by culturing cells in nutrient-deprived medium (NDM) as previously described [9]. Briefly, the NDM composition was 265 mg/L $CaCl_2{\cdot}H_2O$, 400 mg/L KCl, 200 mg/L $MgSO_4{\cdot}7H_2O$, 6400 mg/L NaCl, 163 mg/L $NaH_2PO_4{\cdot}2H_2O$, 0.1 mg/L $Fe(NO_3)_3{\cdot}9H_2O$, 5 mg/L phenol red, 100,000 U/L penicillin G, 100 mg/L streptomycin, 25 mmol/L HEPES buffer (pH 7.4), and MEM vitamin solution (Invitrogen, Carlsbad, CA); the final pH was adjusted to 7.4 with 10% $NaHCO_3$.

Preferential cytotoxicity in nutrient-deprived conditions. PANC-1 cells (2.5×10^4 cells/well) in 96-well plates were cultured in DMEM (10% FBS) for 24 h. The cells were washed with PBS and the medium was replaced with either fresh DMEM (10% FBS) or NDM (−). Test samples were added to the well and cells were cultured for 24 h. Furthermore, the medium was replaced with DMEM (10% FBS) containing 0.5 mg/mL thiazolyl blue tetrazolium bromide (MTT; Sigma–Aldrich) and incubated for 3 h to determine cytotoxicity using the MTT assay [19]. Hypoxia was achieved by culturing cells with a mixture of 1% O_2, 5% CO_2 and 94% N_2.

Measurement of cellular ATP content. PANC-1 cells (2.5×10^4 cells/well) in 96-well plates were cultured in DMEM (10% FBS) for 24 h. The cells were washed with PBS and cultured in fresh DMEM (10% FBS) or NDM (−) with 0.25 μmol/L rotenone, 0.27 μmol/L atpenin A_5, 0.10 μmol/L antimycin A or 0.06 μmol/L efrapeptin F for 24 h. The ATP level in cells was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI).

Flow cytometric analysis. PANC-1 cells (5×10^5) in 60-mm dishes were incubated in DMEM (10% FBS) for 24 h. The cells were washed with PBS and the medium was replaced with either fresh DMEM (10% FBS) or NDM (−). Mitochondrial inhibitors (0.1 μmol/L) were added to the well and the cells were cultured for 24 h. The cells were incubated with annexin V-FITC and propidium iodide according to an annexin V-FITC apoptosis detection kit (Biovision Research Products, Mountain View, CA) and analyzed using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

Animal experiments. Male severe combined immunodeficient (SCID) mice, 6 weeks old, were purchased from Charles River Japan (Yokohama, Japan) and maintained in a specific pathogen-free barrier facility according to our institutional guidelines. PC-3 cells (1×10^7) were subcutaneously injected into the SCID mouse in the left lateral flank. Five days after inoculation, mice were divided randomly into test groups (control $n = 9$, efrapeptin F-treated $n = 7$) and efrapeptin F was intravenously administered twice weekly for 3 weeks to the efrapeptin F-treated group. Cisplatin was intravenously administered once weekly for 3 weeks. Tumor volume was estimated using the following formula: tumor volume (mm^3) = (length × width^2)/2.

Statistical analysis. All data are representative of three independent experiments with similar results. The statistical data are expressed as mean ± SD using descriptive statistics. Statistical analysis was done by using Student's *t*-test.

Results

Efrapeptin F is preferentially cytotoxic to cancer cells in nutrient-deprived conditions

To identify cytotoxic agents that function preferentially on nutrient-deprived cancer cells, we screened the cultured media from various microorganisms. One extract of microbial cultured media exhibited preferential cytotoxicity to PANC-1 cells in nutrient-deprived medium (NDM (−)). The extract was subjected to chromatography to obtain a pure compound. The NMR and MS spectra data revealed its chemical structure to be efrapeptin F (Fig. 1A) [15,16]. Efrapeptin F exhibited preferential cytotoxicity to PANC-1 cells in NDM (−), but not in nutrient-sufficient medium (DMEM (10% FBS)) (Fig. 1B). The cytotoxic effect of efrapeptin F on PANC-1 cells in NDM (−) (IC_{50} = 0.052 μmol/L) was more than 100 times stronger than in DMEM (10% FBS) (IC_{50} = >10 μmol/L).

Mitochondrial inhibitors are preferentially cytotoxic to cancer cells in nutrient-deprived conditions

Efrapeptin F has been previously reported to act as an inhibitor of mitochondrial F_1F_0-ATPase (complex V) [17]. Therefore, we examined whether mitochondrial complex V inhibitors function as cytotoxic agents preferentially on nutrient-deprived cells (Fig. 1B). Interestingly, leucinostatin A and oligomycin (complex V inhibitors) were more cytotoxic to PANC-1 cells in NDM (−) compared with DMEM (10% FBS) [20,21]. In addition, rotenone and piericidin A_1 (NADH-ubiquinone reductase (complex I) inhibitors), atpenin A_5 (a succinate-ubiquinone reductase (complex II) inhibitor), antimycin A, stigmatellin and myxothiazol (ubiquinone-cytochrome *c* (complex III) inhibitors) also were more cytotoxic to PANC-1 cells in NDM (−) compare to DMEM (10% FBS) (Fig. 1C) [20–22]. These results clearly demonstrate that mitochondrial inhibitors exhibit preferential cytotoxicity to nutrient-deprived PANC-1 cells. Efrapeptin F (a complex V inhibitor), rotenone (a complex I inhibitor), atpenin A_5 (a complex II inhibitor), and antimycin A (a complex III inhibitor) were selected for further study. The mode of cell death caused by mitochondrial inhibitors in nutrient-deprived conditions was examined using annexin V-FITC and propidium iodide double staining and flow cytometry. Mitochondrial inhibitors significantly increased the early-apoptotic and late-apoptotic cells in nutrient-deprived conditions, but not to nutrient-sufficient conditions (Fig. 1D). These results suggested that these inhibitors induce apoptosis in nutrient-deprived cells.

Mitochondrial inhibitors are preferentially cytotoxic to cancer cells only under glucose-limiting conditions

To determine what nutrient component was responsible for cytotoxicity of mitochondrial inhibitors, we examined the effect

Fig. 2. Effect of mitochondrial inhibitors on PANC-1 survival under glucose-starved conditions and hypoxic conditions. (A) Effect of nutrient starvation on cytotoxicity of mitochondrial inhibitors. PANC-1 cells were incubated with inhibitors in nutrient-deprived medium containing glucose, amino acids and/or dialyzed FBS for 24 h. (B) Effect of glucose levels on cytotoxicity of mitochondrial inhibitors. PANC-1 cells were incubated with inhibitors in DMEM (10% dialyzed FBS) containing the indicated concentrations of glucose for 24 h. (C) Effect of hypoxia on cytotoxicity of mitochondrial inhibitors. PANC-1 cells were incubated with inhibitors in DMEM (10% FBS) (●) or NDM (−) (○) under 1% O_2 for 24 h.

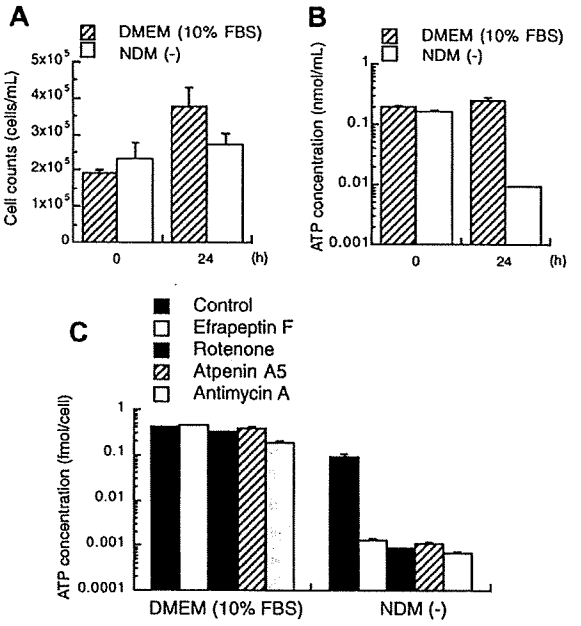


Fig. 3. Effect of mitochondrial inhibitors on cellular ATP levels of PANC-1 cells grown in nutrient-deprived medium. (A) Effect of nutrient starvation on PANC-1 cell growth. PANC-1 cells were incubated in DMEM (10% FBS) or NDM (−) for 24 h and cell numbers were measured by cell counting. (B) Cellular ATP levels were determined by the CellTiter-Glo Luminescent Cell Viability Assay after incubation in DMEM (10% FBS) or NDM (−) for 24 h. (C) PANC-1 cells were incubated with 0.25 μmol/L rotenone, 0.27 μmol/L atpenin A_5, 0.10 μmol/L antimycin A and 0.06 μmol/L efrapeptin F in DMEM (10% FBS) or NDM (−) for 24 h and cellular ATP levels were determined.

of these inhibitors on PANC-1 cell survival under various nutrient-starved conditions (Fig. 2A). Mitochondrial inhibitors preferentially induced cell death under glucose-deprived conditions, irrespective of the presence or absence of amino acids and/or serum. We then examined the effect of glucose levels on cytotoxicity of these inhibitors (Fig. 2B). The concentration of glucose in DMEM is 1000 mg/L. Mitochondrial inhibitors did not induce cell death in the PANC-1 cells cultured with 1000 and 500 mg/L glucose, but in less than 100 mg/L glucose each inhibitor exhibited cytotoxicity. These results demonstrate clearly that glucose is the key component to determine the sensitivity of cancer cells to mitochondrial inhibitors.

Mitochondrial inhibitors are preferentially cytotoxic to nutrient-deprived cells under hypoxic conditions

Because large areas of tumor are exposed not only to nutrient starvation but also to hypoxic conditions, we examined preferential cytotoxicity of mitochondrial inhibitors to nutrient-deprived cells in hypoxic conditions (Fig. 2C). These inhibitors were more cytotoxic to nutrient-deprived PANC-1 cells in 1% O_2 as well as 21% O_2. Our results demonstrate that mitochondrial inhibitors show preferentially cytotoxicity to nutrient-deprived cells not only under normoxic conditions but also under hypoxic conditions.

Reduction of cellular ATP levels by mitochondrial inhibitors induces preferential cell death to nutrient-deprived cells

To investigate why mitochondrial inhibitors exhibit preferential cytotoxicity to nutrient-deprived cells, we examined the effect of mitochondrial inhibitors on cellular ATP levels in nutrient-deprived cells. When PANC-1 cells were incubated in NDM (−) for 24 h, the cells grew less and the cellular ATP levels were markedly decreased (Fig. 3A and B). Since PANC-1 cells incubated in NDM (−) for 24 h could hardly be stained by trypan blue, the cells were able to survive in nutrient starvation in spite of lower ATP levels (Fig. S1). When PANC-1 cells were exposed to mitochondrial inhibitors for 24 h, the amount of cellular ATP were slightly decreased in DMEM (10% FBS), whereas in NDM (−) cellular ATP content decreased 100-fold compared to controls (Fig. 3C). These results indicate that depletion of ATP exerts preferential cytotoxicity to nutrient-starved cells.

Table 1
Growth inhibitory activity of efrapeptin F against 39 human cancer cell lines in the JFCR39 panel.

Origin of cancer	Cell line	Log GI_{50} (μmol/L)[a]
Breast	HBC-4	−7.22
	BSY-1	−6.73
	HBC-5	−8.00
	MCF-7	−8.00
	MDA-MB-231	−5.94
Central nervous system	U251	−7.45
	SF-268	−6.17
	SF-295	−8.00
	SF-539	−6.13
	SNB-75	−5.79
	SNB-78	−6.47
Colon	HCC2998	−6.84
	KM-12	−6.65
	HT-29	−6.86
	HCT-15	−5.61
	HCT-116	−6.48
Lung	NCI-H23	−8.00
	NCI-H226	−6.60
	NCI-H522	−8.00
	NCI-H460	−6.69
	A549	−6.53
	DMS273	−6.64
	DMS114	−8.00
Melanoma	LOX-IMVI	−6.71
Ovary	OVCAR-3	−6.58
	OVCAR-4	−5.85
	OVCAR-5	−6.21
	OVCAR-8	−8.00
	SK-OV-3	−6.46
Kidney	RXF-631L	−5.17
	ACHN	−5.94
Stomach	St-4	−6.10
	MKN1	−6.56
	MKN7	−8.00
	MKN28	−8.00
	MKN45	−6.78
	MKN74	−8.00
Prostate	DU-145	−6.76
	PC-3	−8.00
MG-MID[b]		−6.87
Delta[c]		1.13
Range[d]		2.83

[a] Log concentration of efrapeptin F for inhibition of cell growth at 50% compared to control.
[b] Mean value of log GI_{50} over all cell lines tested.
[c] The difference in log GI_{50} value of the most sensitive cell and MG-MID value.
[d] The difference in log GI_{50} value of the most sensitive cell and the least sensitive cell.

Efrapeptin F inhibits tumor growth in vivo

PANC-1 cells are low tumorigenicity even in immunodeficient mice. To explore the *in vivo* antitumor activity of mitochondrial inhibitors, we examined the growth inhibitory activity of efrapeptin F against 39 human cancer cell lines of the JFCR39 panel (Table 1) [23–25]. Efrapeptin F exhibited potent growth inhibitory

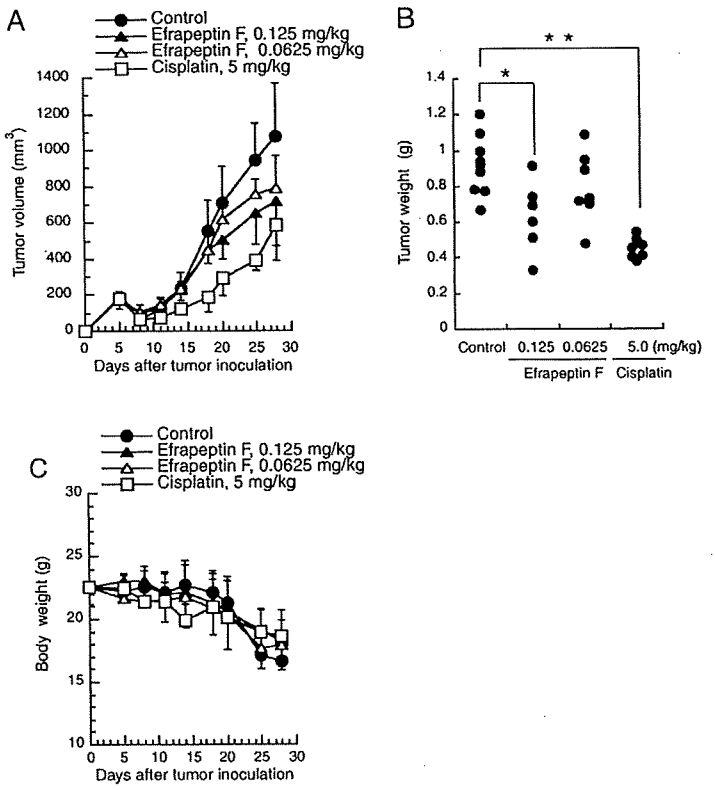


Fig. 4. Antitumor effect of efrapeptin F on PC-3 cells in SCID mice. PC-3 cells (1×10^7) were subcutaneously inoculated into SCID mice on day 0. Efrapeptin F was administrated intravenously twice weekly for 3 weeks from day 5. (A) Tumor volumes. *Y* axis, tumor volume (mm^3); *X* axis, time (day). (B) Tumor weight. The tumors were excised on day 28. $^{**}P < 0.001$; $^{*}P < 0.05$, compared with control (Student's *t*-test). (C) Body weight. *Y* axis, body weight (g); *X* axis, time (day). *Points*, mean values; *bars*, SD.

activity, and the mean value for log concentration for inhibition of cell growth at 50% compared to control was −6.87 (135 μmol/L). In particular, HBC-5, MCF-7, SF-295, NCI-H23, NCI-H522, DMS114, OVCAR-8, MKN7, MKN28, MKN74 and PC-3 cells were sensitive to efrapeptin F. Efrapeptin F showed preferential cytotoxicity to PC-3 cells in nutrient-deprived conditions as well as to PANC-1 cells (Fig. S2). Therefore, xenograft models of PC-3 cells were used to evaluate the *in vivo* antitumor activity of efrapeptin F. Efrapeptin F was intravenously administered twice weekly for 3 weeks from day 5 after the tumor inoculation. Efrapeptin F inhibited tumor growth of the PC-3 xenograft (Fig. 4A and B). Efrapeptin F at 0.125 and 0.0625 mg/kg reduced tumor weight by 68% and 86%, respectively (Tumor weight (g), control = 0.92 ± 0.17 (mean ± SD), 0.125 mg/kg efrapeptin F = 0.63 ± 0.20, 0.0625 mg/kg efrapeptin F = 0.79 ± 0.20) (Fig. 4B). To assess toxicity, we measured the body weight of the tumor-bearing mice (Fig. 4C). Their weight was not reduced by administration of efrapeptin F at these doses. However, among seven mice that were administrated efrapeptin F at a high dose (125 μg/kg), only one mouse died at day 23. Remaining mice survived until the end of the experiment without a decrease of body weight and anatomically without toxic effects in critical organs.

Discussion

Tumor microenvironment strongly affects tumor development and progression. Many aspects of physiology that differentiate solid tumors from normal tissues arise from differences in vasculature. Disorganized vascular systems in tumors result in large areas of tumor exposed to nutrient starvation and hypoxic conditions. In addition, due to the unregulated growth of tumor cells caused by genetic and epigenetic alterations, tumor cells proliferate more rapidly than normal cells and nutrient and oxygen demands often exceed supply [26–28]. In particular, highly aggressive tumor cells such as pancreatic cancers that are relatively hypovascular, are able to survive even in conditions of low nutrients and low oxygen supply. Since chronic nutrient deprivation seldom occurs in normal tissues, one strategy for anticancer agent development is to target cancer cells growing in nutrient-deprived conditions. Thus, we screened to identify cytotoxic agents that function preferentially in nutrient-deprived cancer cells.

Previous studies have shown that conventional chemotherapeutic drugs and various small molecule inhibitors were only weakly cytotoxic to cancer cells in nutrient-deprived conditions [9]. In this study, we found that the small molecule efrapeptin F, which is produced by some fungi showed preferential cytotoxicity to PANC-1 cells grown in nutrient-deprived conditions compared with cells in nutrient-sufficient conditions. Because efrapeptin F inhibits the mitochondrial complex V, we examined whether mitochondrial complex V inhibitors such as leucinostatin A and oligomycin act as cytotoxic agents preferentially on nutrient-deprived cells. Interestingly, these inhibitors were more cytotoxic to PANC-1 cells in NDM (−) compared to DMEM (10% FBS). In addition, mitochondrial complex I inhibitors (rotenone and piericidin A_1), a complex II inhibitor (atpenin A_5), and complex III inhibitors (antimycin A, stigmatellin and myxothiazol) also were more cytotoxic to PANC-1 cells in NDM (−). These results clearly demonstrate that mitochondrial inhibitors exhibit preferential cytotoxicity to nutrient-deprived PANC-1 cells, suggesting that mitochondrial inhibitors have unique and attractive characteristics in antitumor agent development. These inhibitors induced cell death under glucose-limiting conditions, irrespective of the presence or absence of amino acids and/or serum. The glucose concentration in colon cancers is only ~1 of 45 of typical plasma glucose concentration (1000 mg/L

or 5.6 nmol/L) [13]. Mitochondrial inhibitors did not induce cell death in 1000 mg/L glucose, but each inhibitors exhibited cytotoxicity in less than 100 mg/L glucose levels. The cytotoxicity caused by mitochondrial inhibitors depended on glucose levels in the culture medium and glucose was the key component to determine the sensitivity of cancer cells to their inhibitors. However, it is unclear how mitochondrial inhibitors exhibit preferential cytotoxicity to nutrient-deprived cells. The cellular ATP level was markedly decreased in PANC-1 cells grown in nutrient starvation. Mitochondrial inhibitors induced ATP depletion in nutrient-deprived cells at lower concentrations of inhibitors compared with nutrient-sufficient cells, thereby these inhibitors could exert preferential cytotoxicity under nutrient-deprived conditions.

Large areas of tumor are exposed not only to nutrient starvation but also hypoxic conditions. Therefore, we examined preferential cytotoxicity of mitochondrial inhibitors to nutrient-deprived cells in hypoxic conditions. Mitochondrial inhibitors showed preferential cytotoxicity to nutrient-deprived cells not only under hypoxic conditions but also under normoxic conditions. Normal tissue uses glycolysis to generate approximately 10% of the cellular ATP, with mitochondria accounting for 90%. In tumor sections, however, over 50% of the cellular ATP is produced by glycolysis with the remainder being generated at the mitochondria [29]. In hypoxic conditions (1% O_2), HIF-1α was stabilized and accumulated in nutrient-deprived PANC-1 cells, and the real-time PCR analysis revealed that hexokinase 2 and glucose transporter-1 expression were increased (data not shown). Despite PANC-1 cells grown in nutrient-deprived and hypoxic conditions were represented activation of glycolysis and induction of glucose transporter-1, mitochondrial inhibitors exhibited strong cytotoxicity to these cells. Therefore, ATP generation by mitochondria appeared to be essential for cell survival under hypoxic as well as normoxic conditions.

PANC-1 cells are low tumorigenicity even in SCID mice. To examine the *in vivo* antitumor activity of mitochondrial inhibitors, we explored cancer cell lines that were more sensitive to efrapeptin F. The growth inhibitory activity of efrapeptin F against 39 human cancer cell lines of the JFCR39 panel revealed that human prostate cancer PC-3 cells were highly sensitive to efrapeptin F. Thus, PC-3 cancer xenograft models were used to evaluate *in vivo* antitumor activity, and efrapeptin F was found to induce regression of PC-3 xenograft tumors. In this study, we demonstrated that mitochondrial inhibitors showed preferential cytotoxicity to nutrient-deprived cancer cells relative to nutrient-sufficient cells. Therefore, the potent cytotoxicity of these inhibitors to cancer cells deprived of nutrients (simulating a tumor microenvironment) makes mitochondria a promising target for new drugs that may be developed to treat a broad spectrum of malignant tumors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.050.

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Inhibitors of insulin-like growth factor-1 receptor tyrosine kinase are preferentially cytotoxic to nutrient-deprived pancreatic cancer cells

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ABSTRACT

Chronic deprivation of nutrients is rare in normal tissues, however large areas of tumor are nutrient-starved and hypoxic due to a disorganized vascular system. Some cancers show an inherent ability to tolerate severe growth conditions. Therefore, we screened chemical compounds to identify cytotoxic agents that function preferentially in nutrient-deprived conditions. We found that AG1024, a specific inhibitor of insulin-like growth factor-1 receptor tyrosine kinase (IGF-1R), showed preferential cytotoxicity to human pancreatic cancer cells in nutrient-deprived conditions relative to cells in nutrient-sufficient conditions. The cytotoxicity of I-OMe-AG538 (another specific inhibitor of IGF-1R kinase) was also enhanced in nutrient-deprived cells. In addition, AG1024 and I-OMe-AG538 potently inhibited IGF-1R activation to nutrient-deprived cells. In contrast, conventional chemotherapeutic drugs, as well as inhibitors of PDGFR and EGFR kinases, elicited weak cytotoxicity. These data indicate that nutrient-deprived human pancreatic cancer cells have increased sensitivity to inhibition of IGF-1R activation. IGF-1R inhibitors offer a promising strategy for anticancer therapeutic approaches that are oriented toward tumor microenvironment.

Patients diagnosed with pancreatic cancer, an aggressive disease with the lowest 5-year survival rates of all cancers, develop metastases rapidly and die within a short period of time after diagnosis [1,2]. Pancreatic cancer is largely resistant to almost all known chemotherapeutic agents, including 5-fluorouracil, paclitaxel and doxorubicin; surgery is the only current treatment modality that offers any prospect of potential cure. Clearly, there is a dire need for new therapeutic alternatives that improve clinical outcome for pancreatic cancer patients.

Tumor microenvironment exerts an important influence on cancer physiology. The disorganized vascular system in a tumor often results in large areas of tumor starved for nutrients and oxygen. Pancreatic cancers in particular, which are characterized as hypovascular tumors, show an inherent ability to tolerate severe growth conditions. Certain human pancreatic cancer cell lines, including PANC-1, AsPC-1, BxPC-3 and KP-3, exhibit marked environmental tolerance and can survive for prolonged periods of time in nutrient-deprived conditions. Because tolerance of these cancer cells to nutrient starvation has been associated with the activity of protein kinase B (PKB)/Akt [3], it has been hypothesized that agents that diminish such tolerance could function as anticancer agents [4–7].

Insulin-like growth factors-1 (IGF-1) and -2 (IGF-2) are involved in the pathophysiology of a wide range of human neoplasias due to the mitogenic and antiapoptotic properties mediated by their type 1 receptor (IGF-1R) [8]. IGF-1R is a tetrameric transmembrane receptor tyrosine kinase composed of two α and β subunits. The extracellular α subunit is responsible for ligand binding, whereas the β subunit consists of a transmembrane domain and an intracellular tyrosine kinase domain [9,10]. Ligand binding activates the intrinsic receptor tyrosine kinase, resulting in trans-β subunit autophosphorylation and stimulation of PI3K-AKT-TOR and RAF-MAPK signaling pathways. In addition to cell proliferation, activation of IGF-1R has been reported to stimulate cell survival, transformation, metastasis and angiogenesis [11]. Targeted inhibition of IGF-1R signaling has been shown to result in impressive anti-neoplastic activity in many *in vitro* and *in vivo* models of common human cancers. IGF-1R small interfering RNAs [12], anti-receptor antibodies [13,14], a IGF-1-like competitive peptide antagonist [15], a dominant-negative IGF-1R [16–18] and small-molecule IGF-1R tyrosine kinase inhibitors [19,20] have all been found to interfere with cell growth and proliferation. IGF-1R is therefore regarded as an attractive potential target in the development of new drugs to treat malignant tumors.

In this study, we screened chemical compounds to identify agents that preferentially reduce the survival of nutrient-deprived human pancreatic cancer PANC-1 cells. The screen identified IGF-1R inhibitors, which function as cytotoxic agents preferentially on human pancreatic cancer cells in nutrient-deprived conditions.

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Materials and methods

Materials. Antibodies used in Western blotting included anti-IGF-1Rβ (sc-713), anti-Erk 1 (sc-93) and anti-phospho-Erk (sc-7383) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-α-tubulin (T5168) from Sigma–Aldrich (St. Louis, MO); and anti-Akt (#9272), anti-phospho-Akt (Ser 473) (#9271), anti-phospho-Akt (Thr 308) (#9275) and anti-phospho-IGF-1R (#3021) from Cell Signaling Technology (Denvers, MA). Recombinant human IGF-1 was from R&D Systems (Minneapolis, MN). AG1024, AG1296, AG1478 and I-OMe-AG538 were obtained from Calbiochem (Madison, WI). Doxorubicin hydrochloride, fluorouracil, paclitaxel and mitomycin C were from Sigma. The SCADS inhibitor kit I consisting of 79 chemical inhibitors with ~60 different targets was kindly provided by the Screening Committee on Anticancer Drugs (Japan).

Cells and culture. Human pancreatic cancer cell lines PANC-1, Capan-1, MIA Paca-2, BxPC-3 and PSN-1 were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA), 100,000 U/L penicillin G, and 100 mg/L streptomycin. Nutrient starvation was achieved by culturing the cells in nutrient-deprived medium (NDM) as previously described [3–7]. Briefly, the composition of the NDM was as follows: 265 mg/L $CaCl_2{\cdot}H_2O$, 400 mg/L KCl, 200 mg/L $MgSO_4{\cdot}7H_2O$, 6400 mg/L NaCl, 163 mg/L $NaH_2PO_4{\cdot}2H_2O$, 0.1 mg/L $Fe(NO_3)_3{\cdot}9H_2O$, 5 mg/L phenol red, 100,000 U/L penicillin G, 100 mg/L streptomycin, 25 mmol/L HEPES buffer (pH 7.4), and MEM vitamin solution (Invitorogen, Carlsbad, CA); the final pH was adjusted to 7.4 with 10% $NaHCO_3$.

Preferential cytotoxicity in nutrient-deprived conditions. PANC-1 cells (2.5×10^4 cells/well) in 96-well plates were cultured in DMEM for 24 h. The cells were washed with PBS and the medium was replaced with either fresh DMEM or NDM. Test samples were added to the well and cells were cultured for 24 h. Cytotoxicity was determined using the MTT assay [21].

Preparation of cell lysate and Western blotting. PANC-1 cells (5×10^5) in 35-mm dishes were incubated in DMEM for 24 h. The cells were washed with PBS and the medium was replaced with either fresh DMEM or NDM. AG1024 or I-OMe-AG538 was added to each dish and the cells were incubated for 1 h prior to stimulation with 50 ng/ml IGF-1 for 10 min. The cells were washed twice with ice-cold PBS containing 100 μm Na_3VO_4 and then lysed in a lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 50 mM NaF, 50 mM β-glycerolphosphate, 1 mM Na_3VO_4, pH 7.5, and 25 μg/ml each of antipain, leupeptin, and pepstatin). Equal amounts of protein extract were separated by SDS–polyacrylamide gel electrophoresis, transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with anti-IGF-1R, anti-phospho-IGF-1R, anti-Akt, anti-phospho-Akt (Thr 308), anti-phospho-Akt (Ser 473), anti-Erk 1,

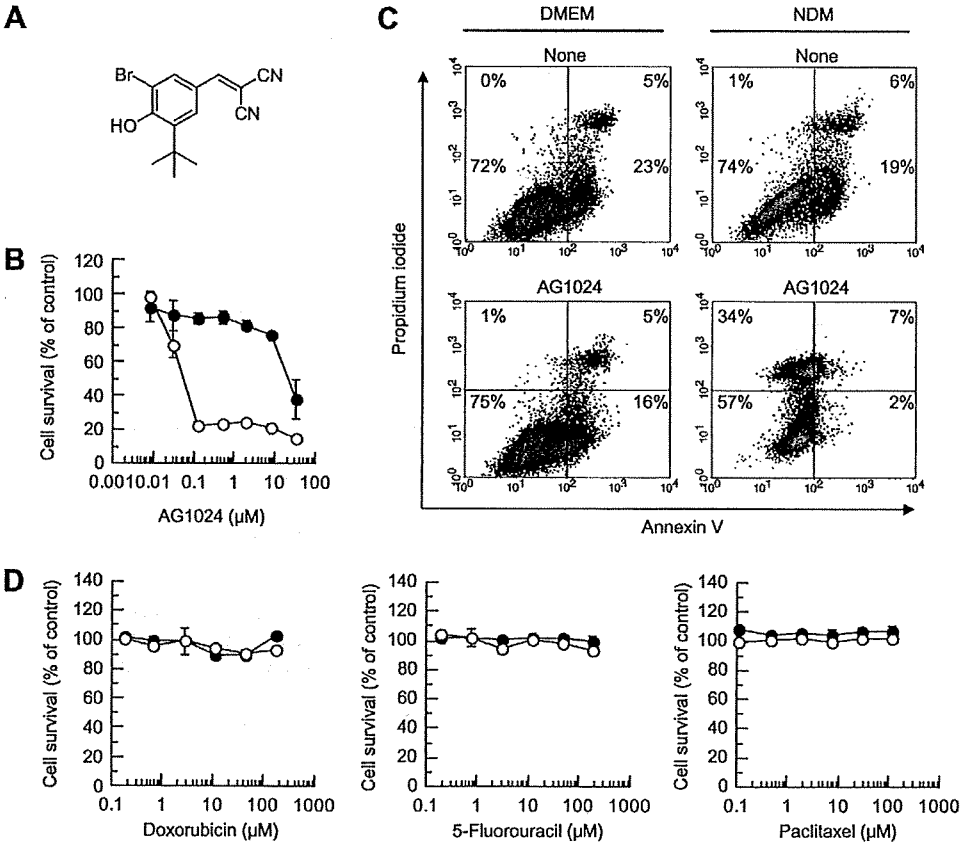

Fig. 1. Effect of AG1024 on survival of PANC-1 cells in nutrient-deprived conditions. (A) Structure of AG1024. (B) Effect of AG1024 on PANC-1 cell viability in normal medium, DMEM (●) and nutrient-deprived medium, NDM (○). PANC-1 cells incubated in DMEM for 24 h. The cells were then washed with PBS and the medium was replaced with either fresh DMEM or NDM. The indicated concentrations of AG1024 were added to each well and the cells were incubated for 24 h. Cell viability was determined using the MTT assay. (C) Flow cytometric analysis of AG1024-treated PANC-1 cells. PANC-1 cells were cultured with 0.3 μM AG1024 in DMEM or NDM for 12 h. The cells were stained with annexin V-FITC and propidium iodide according to instructions for the apoptosis detection kit and then analyzed using a flow cytometer. (D) Effect of conventional anticancer drugs on survival of PANC-1 cells in nutrient-deprived conditions. PANC-1 cells were incubated with indicated concentrations of doxorubicin, 5-fluorouracil and paclitaxel in DMEM (●) or NDM (○) for 24 h.

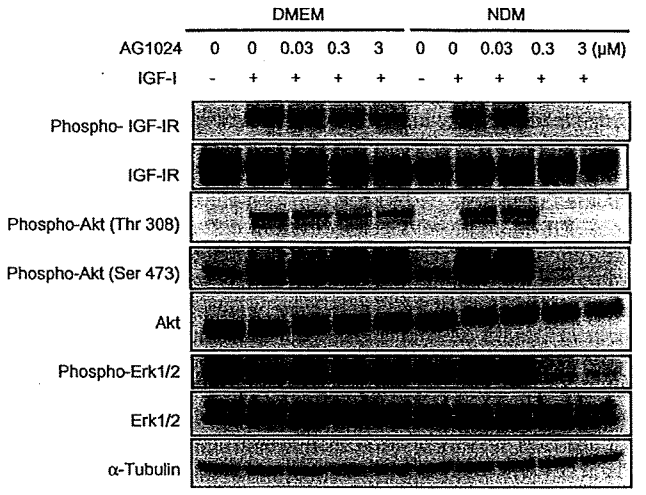

Fig. 2. Effect of AG1024 on IGF-1R activation. PANC-1 cells incubated in DMEM for 24 h were washed with PBS and the medium was replaced with either fresh DMEM or NDM. The cells were incubated with the indicated concentration of AG1024 for 1 h before stimulation with 50 ng/ml IGF-1 for 10 min. Cell lysates were resolved using SDS–PAGE and transferred to membranes for western blotting with specific antibodies.

anti-phospho-Erk, or anti-tubulin antibodies. Horseradish peroxidase-linked anti-rabbit IgG or anti-mouse IgG antibodies were used as secondary antibodies (GE Healthcare, Piscataway, NJ). The blots were developed using ECL reagent according to the manufacturer's instructions (GE healthcare).

Flow cytometric analysis. PANC-1 cells (5×10^5) in 60-mm dishes were incubated in DMEM for 24 h. The cells were washed with PBS and the medium was replaced with either fresh DMEM or NDM. AG1024 (0.3 μM) was then added and the cells were cultured for 12 h. The cells were incubated with annexin V-FITC and propidium iodide using an annexin V-FITC apoptosis detection kit (Biovision Research Products, Mountain View, CA) and analyzed using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

Statistical analysis. All data are representative of three independent experiments with similar results. The statistical data are expressed as mean ± SD using descriptive statistics.

Results

AG1024 is preferentially cytotoxic to human pancreatic cancer PANC-1 cells in nutrient-deprived conditions

To identify cytotoxic agents that function preferentially on nutrient-deprived cells, we tested the cytotoxic effects of small-molecule inhibitors in the SCADS inhibitors kit I. As shown in Table S1, a specific inhibitor of IGF-1R tyrosine kinase, termed AG1024, was found to be cytotoxic to PANC-1 cells in nutrient-deprived medium (NDM), but not in normal medium (DMEM). The structure of AG1024 [22], otherwise known as 2-(3-bromo-5-t-butyl-4-hydroxybenzylidene)malonitrile, is shown in Fig. 1A. To determine the dose–response relationship of AG1024 cytotoxicity, PANC-1

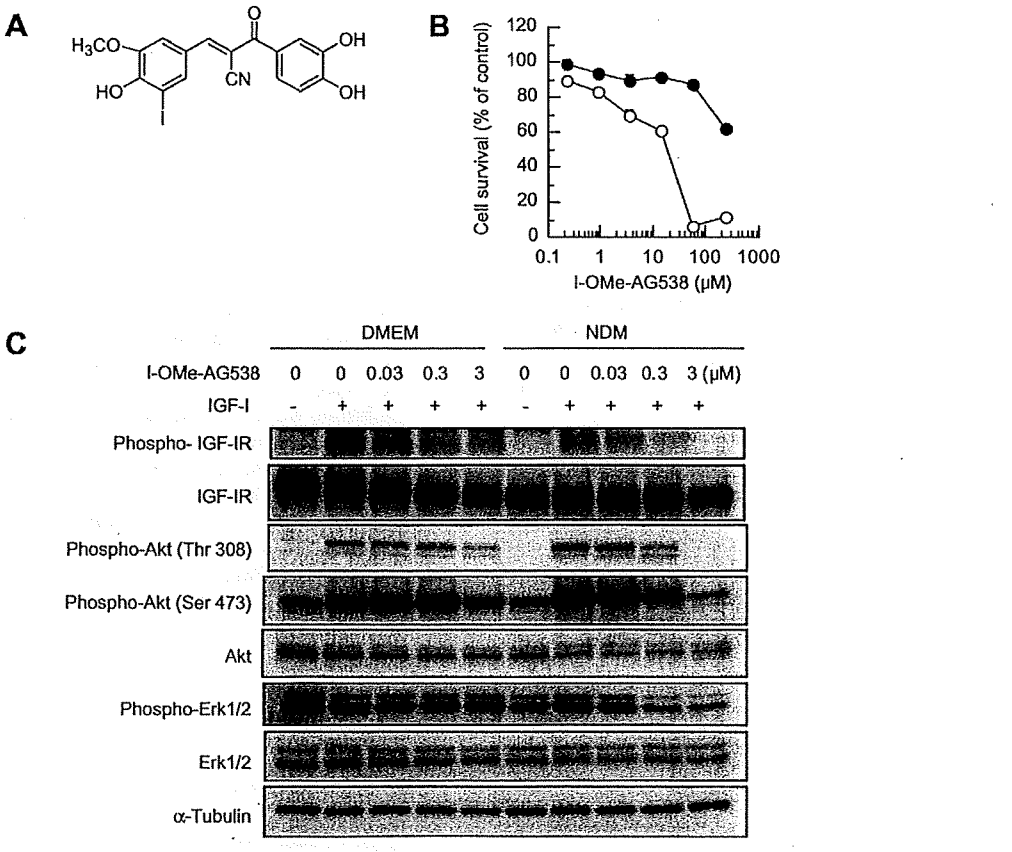

Fig. 3. Effect of I-OMe-AG538 on survival of nutrient-deprived PANC-1 cells. (A) Structure of I-OMe-AG538. (B) Effect of I-OMe-AG538 on PANC-1 cell viability in DMEM (●) or NDM (○). PANC-1 cells were incubated with the indicated concentrations of I-OMe-AG538 in DMEM or NDM for 24 h. (C) Effect of I-OMe-AG538 on IGF-1R activation. PANC-1 cells in DMEM or NDM were incubated with the indicated concentration of I-OMe-AG538 for 1 h before stimulation with 50 ng/ml IGF-1 for 10 min. Cell lysates were resolved using SDS–PAGE and transferred to membranes for western blotting with specific antibodies.

cells grown in NDM or DMEM were exposed to increasing concentrations of AG1024 for 24 h (Fig. 1B). The cytotoxic effect of AG1024 was more than 100 times greater on nutrient-deprived PANC-1 cells (NDM IC_{50} 0.055 μM) relative to cells in nutrient-sufficient medium (DMEM IC_{50} 21 μM). In DMEM, 0.3 μM AG1024 did not induce any significant PANC-1 cell death as determined using propidium iodide and annexin V staining and flow cytometry (Fig. 1C). In contrast, 34% of the cells grown in NDM and treated with the same concentration of AG1024 showed propidium iodide-positive/annexin V-negative staining. We compared the cytotoxicity of AG1024 to that of several conventional anticancer drugs, including doxorubicin, 5-fluorouracil and paclitaxel, in PANC-1 cells grown in NDM versus DMEM (Fig. 1D). The cytotoxicity of doxorubicin, 5-fluorouracil and paclitaxel on PANC-1 cells grown in either medium for 24 h was significantly weaker than AG1024. These results demonstrate clearly that AG1024 exhibits preferential cytotoxicity to nutrient-deprived PANC-1 cells.

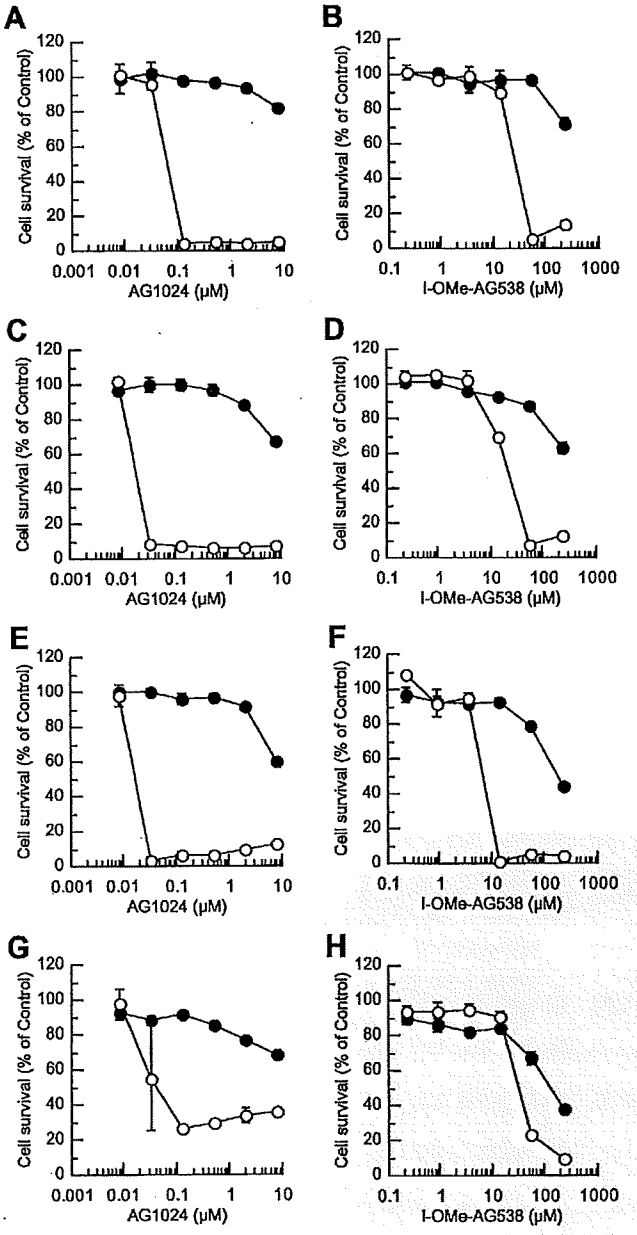

Fig. 4. Effect of AG1024 and I-OMe-AG538 on various human pancreatic cancer cell lines in nutrient-deprived conditions. Human pancreatic cancer cells were incubated with indicated concentrations of AG1024 or I-OMe-AG538 in DMEM (●) or NDM (○) for 24 h. A,B, Capan-1; C,D, MIA Paca-2; E,F, BxPC-3; G,H, PSN-1.

AG1024 inhibits activation of IGF-1R in nutrient-deprived PANC-1 cells

Because AG1024 is a specific inhibitor of IGF-1R kinase, we examined the effect of AG1024 on IGF-1-mediated phosphorylation of IGF-1R in PANC-1 cells grown in different media (Fig. 2). While addition of 0.3 μM AG1024 to PANC-1 cells grown in NDM resulted in a complete inhibition of IGF-1R autophosphorylation, phosphorylation of IGF-1R was only weakly inhibited in cells grown in DMEM with 10-fold higher concentrations of AG1024 (3 μM). AG1024 also inhibited the phosphorylation of Akt (Thr 308), Akt (Ser 473) and Erk, which normally occur as a result of IGF-1R activation. These results demonstrate that AG1024 is a potent inhibitor of IGF-1R activation in nutrient-deprived PANC-1 cells.

I-OMe-AG538 is preferentially cytotoxic to nutrient-deprived PANC-1 cells

In testing whether other IGF-1R inhibitors also functioned preferentially in nutrient-deprived cells, we found that I-OMe-AG538 [23] (another specific inhibitor of IGF-1R, Fig. 3A) also was more cytotoxic to cells in nutrient-deprived medium relative to those in nutrient-sufficient conditions (Fig. 3B). The effect of I-OMe-AG538 on IGF-1R activation in nutrient-deprived cells was similar to AG1024, in that it blocked phosphorylation of IGF-1R, Akt and Erk (Fig. 3C). Our results demonstrate clearly that the IGF-1R inhibitors AG1024 and I-OMe-AG538 both inhibit IGF-1R-mediated signaling and are preferentially cytotoxic to nutrient-deprived PANC-1 cells.

Inhibitors of IGF-1R show preferential cytotoxicity to various human pancreatic cancer cells lines in nutrient-deprived conditions

To determine whether inhibitors of IGF-1R kinase exhibit preferential cytotoxicity to other nutrient-deprived human pancreatic cancer cell lines, we examined the cytotoxic effects of AG1024 and I-OMe-AG538 on Capan-1, MIA Paca-2, BxPC-3, and PSN cells (Fig. 4). AG1024 and I-OMe-AG538 were significantly more cytotoxic to all four human pancreatic cancer cell lines in NDM relative to DMEM, indicating that the cytotoxicity of IGF-1R kinase inhibitors is likely to occur in nutrient-deprived human pancreatic cancer cells. To understand the specificity of IGF-1R kinase inhibitors, we also examined the cytotoxic effects of other representative receptor tyrosine kinase inhibitors (Fig. S1). The cytotoxicities of AG1296 (a PDGFR kinase inhibitor) [24] and AG1478 and PD168393 (EGFR kinase inhibitors) [25–27] were significantly reduced, relative to IGF-1R inhibitors, in both nutrient-deprived and -fed PANC-1 cells. These results indicate that specific inhibition of IGF-1R kinase is important in promoting preferential cytotoxicity in nutrient-starved human pancreatic cancer cells.

Discussion

Tumor microenvironment strongly influences tumor growth and progression. Many aspects of physiology that differentiate solid tumors from normal tissues arise from differences in vasculature. Disorganized vascular systems in tumors result in large areas of tumor exposed to nutrient starvation and hypoxic conditions. In addition, due to the unregulated growth of tumor cells caused by genetic and epigenetic alterations, cells proliferate more rapidly than normal cells and nutrient and oxygen demands often exceeds supply [28–30]. Cancer cells, in particular highly aggres-

sive tumors such as pancreatic cancer that are relatively hypovascular, are able to survive even in conditions of low nutrients and low oxygen supply. Since chronic nutrient deprivation seldom occurs in normal tissues, one strategy for anticancer agent development is to target cancer cells growing in nutrient-deprived conditions. Thus, we screened to identify cytotoxic agents that function preferentially in nutrient-deprived cells.

We found that AG1024, a specific inhibitor of IGF-1R kinase, showed preferential cytotoxicity to human pancreatic cancer PANC-1 cells grown in nutrient-deprived medium. Conventional chemotherapeutic drugs such as doxorubicin, 5-fluorouracil and paclitaxel, were only weakly cytotoxic to nutrient-deprived PANC-1 cells, suggesting that AG1024 may be a unique and attractive starting compound in the development of an antitumor agent. AG1024 has been reported to induce apoptosis in human breast cancer MCF-7 cells [31]. In our present study, flow cytometric analysis showed that AG1024 increased propidium iodide staining without annexin V of nutrient-deprived PANC-1 cells. Kigamicin D and (6a*R*,11a*R*)-3,8-dihydroxy-9-methoxypterocarpan induced necrosis in nutrient-deprived cells [4,32]. Therefore, AG1024 may induce necrosis under nutrient starvation. I-OMe-AG538, another IGF-1R kinase inhibitor that differs in structure from AG1024, was also cytotoxic to nutrient-deprived PANC-1 cells. These IGF-1R kinase inhibitors also were cytotoxic to other nutrient-deprived human pancreatic cancer cell lines, including Capan-1, MIA Paca-2, BxPC-3 and PSN.

IGF-1 binding to the IGF-1R results in activation of receptor tyrosine kinases that stimulates signaling through intracellular networks, including PI3K-AKT-TOR and RAF-MAPK, which then promote cell proliferation and inhibit apoptosis. We found that the IGF-1R kinase inhibitors AG1024 and I-OMe-AG538 blocked phosphorylation of IGF-1R by IGF-1 preferentially in cells cultured in nutrient-deprived conditions relative to those in nutrient-sufficient conditions. These IGF-1R kinase inhibitors also suppressed phosphorylation of Akt and Erk, demonstrating that activation of pathways downstream of the IGF-1R were also blocked in nutrient-deprived conditions.

Unlike AG1296 (a PDGFR kinase inhibitor) or AG1478 and PD168393 (EGFR kinase inhibitors), which are less cytotoxic in nutrient-deprived PANC-1 cells, preferential inhibition of IGF-1 signaling by IGF-1R kinase inhibitors suggests that this pathway may play an important role in cell survival in stress conditions such as nutrient deprivation. The Akt pathway, which functions downstream of IGF-1R, plays a critical role in the proliferation, survival, motility, morphology and therapeutic resistance of cancer cells [33,34]. Because Akt has been demonstrated to regulate cell survival in various stress conditions, including nutrient deprivation, this kinase is viewed as a promising target for cancer therapeutics. Akt inhibitors have been developed including PX-316, which shows antitumor activity against human MCF-7 breast cancer and HT-29 colon cancer xenografts in mice [35]. Thus, part of the preferential cytotoxicity of IGF-1R kinase inhibitors in nutrient-deprived conditions may be due to inhibition of Akt activation.

The IGF-1 receptor is universally expressed in various hematologic and solid tumor cells. NVP-ADW742, another specific inhibitor of IGF-1R kinase, has been shown to be a significant antitumor agent in an orthotopic xenograft multiple myeloma model [20]. Oral administration of the IGF-1R kinase-specific inhibitor NVP-AEW541 has been shown to inhibit IGF-1R signaling in tumors and to reduce tumor growth in a xenograft fibrosarcoma model [19]. The potent cytotoxicity of AG1024 and I-OMe-AG538 to pancreatic cancer cell lines deprived of nutrients (simulating a tumor microenvironment) makes IGF-1R a promising target for new drugs that may be developed to treat a broad spectrum of malignant tumors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.01.065.

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