glucose-deprived condition was achieved by culturing the cells in glucose-free medium (Sigma, St. Louis, MO, USA). A hypoxic condition was achieved by incubating the cells in a hypoxia incubator in the presence 5% CO_2 and 1% O_2. The experiments were performed using PANC-1 cells, unless stated otherwise.

Reagents. Gemcitabine (Gemzar; Eli Lilly Co., Indianapolis, IN, USA) and 5-fluorouracil (Kyowa Hakko Kirin Co., Ltd, Tokyo, Japan) were dissolved in saline and stored at −20°C. Cisplatin (Sigma) was dissolved in DMSO on the day of use. UCN-01 was kindly provided by Kyowa Hakko Kirin Co., Ltd. LY294002 and Gö6976 were purchased from Calbiochem (San Diego, CA, USA). Antibodies were purchased from the following manufacturers: anti-total Akt, anti-phosphospecific Akt (Ser 473), anti-phosphospecific Cdc25c (Ser216), anti-phosphor specific Chk1 (Ser345), anti-phosphospecific Chk2 (Thr68), and anti-γ-H2AX (Ser139) from Cell Signaling Technology (Danvers, MA, USA); anti-HIF-1α and anti-HIF-2α antibodies from Novus Biologicals (Littleton, CO, USA); Chk1 (G-4) and Actin (C-11) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Chk2 antibody clone7 from Upstate Biotechnology (Lake Placid, NY, USA). The following secondary antibodies were purchased from Santa Cruz Biotechnology: goat antimouse IgG-HRP, goat antirabbit IgG-HRP, and donkey antigoat IgG-HRP.

Cytotoxicity assay of anticancer drugs. The cytotoxicity assay was performed using Cell Counting kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan), as described previously.[28] The cell number in the absence of anticancer drugs under each culture condition was set as 100%. Values shown represent the means ± SD (n = 4–8).

siRNA transfection. SMARTpool HIF-1α, HIF-2α, Chk1, Chk2 and non-silencing siRNA were purchased from Dharmacon (Lafayette, CO, USA). Cells were seeded at 10^6 cells per dish in 10 mm dishes. At 24 h after seeding, siRNA was added at a final concentration of 100 nM, followed by incubation for 24 h. The knockdown efficacies were determined by Western blot analysis.

Western blot analysis. Protein extraction and Western blot analysis were performed as described previously.[29] The antibody dilutions used were in accordance with the manufacturers' instructions.

Cell cycle analysis. After 24 h preincubation, 1×10^6 cells were cultured in a 60-mm cell culture dish under either normoglycemic/normoxic or hypoglycemic/hypoxic conditions for 24 h, followed by staining using the Click-iT EdU Alexa Fluor 488 Cell Proliferation Assay kit (Molecular Probes, Eugene, OR, USA) in accordance with the manufacturer's instructions, and analyzed on a FACSCalibur (BD Bioscience, San Jose, CA, USA).

DNA ploidy assay. After 24 h preincubation, 1×10^6 cells were cultured in a 60-mm cell culture dish under either normoglycemic/normoxic or hypoglycemic/hypoxic conditions in the presence or absence of 1 μM gemcitabine for 24 h, followed by staining with propidium iodide (Molecular Probes) in accordance with the manufacturer's instruction, and analyzed on a FACSCalibur.

[^{3}H]-Gemcitabine and [^{3}H]-thymidine uptake. After 24 h preincubation, 1×10^6 cells were cultured in a 60-mm cell culture dish under either normoglycemic/normoxic or hypoglycemic/hypoxic conditions for 24 h, followed by incubation for another 3 h with 1 μM [^{3}H]-labeled gemcitabine (6.8 μCi/nmol; Moravek Biochemicals, Brea, CA, USA). The cells were washed thrice with complete medium containing 100 μM gemcitabine, and twice with ice-cold PBS. The cells were detached by trypsinization and counted by the Trypan blue exclusion method. The total cellular uptake of [^{3}H]-gemcitabine was measured by lysing a 10 μL aliquot of the cell suspension and counting the total cell-associated radioactivity using a multipurpose scintillation counter, LS6500 (Beckman Coulter Inc., Fullerton, CA, USA). The incorporation of [^{3}H]-gemcitabine into the DNA was determined by a previously published method, with slight modification.[30]

Statistical analysis. All the results were expressed as the mean ± SD. The statistical analysis was conducted using the Student t-test after an ANOVA.

Results

Effect of the culture condition on the sensitivity to various anticancer drugs. In the first set of experiments, we determined whether hypoxia and hypoglycemia might affect the sensitivity of the cancer cells to gemcitabine, 5-fluorouracil and cisplatin, which are commonly used drugs for systemic chemotherapy of cancer. Pancreatic cancer-derived PANC-1 cells were treated with serial dilutions of anticancer drugs and incubated under either a normoglycemic (1.0 g/L glucose) or hypoglycemic (0 g/L glucose) condition and normoxic (21% O_2) or hypoxic (1% O_2) condition. The 50% inhibitory concentration (IC_{50}) of gemcitabine for the PANC-1 cells incubated under the normoglycemic/normoxic condition was 300 nM, whereas the IC_{50} values of gemcitabine under the hypoxic and hypoglycemic condition were >300 μM, which was 1000 times higher than the value under the normoglycemic/normoxic condition (Fig. 1A). Similarly, the IC_{50} of 5-fluorouracil was greatly influenced by the culture condition, with IC_{50} values of 2.7 μM under the normoglycemic/normoxic condition, 9.6 μM under the hypoglycemic/normoxic condition, 92 μM under the normoglycemic/hypoxic condition, and 79 μM under the hypoglycemic/hypoxic condition (Fig. 1B); the corresponding values for cisplatin were 74, 106, 108 μM, and more than 300 μM (Fig. 1C). The cytotoxicities of gemcitabine for other pancreatic cancer cell lines, PSN-1 and Capan-1, were also examined. The IC_{50} of gemcitabine for the PSN-1 cells was 0.22 μM under the normoglycemic/normoxic condition and more than 300 μM under the hypoglycemic/hypoxic condition (Fig. 1D). The IC_{50} of gemcitabine for the Capan-1 cells was 0.24 μM under the normoglycemic/normoxic condition, and 57 μM under the hypoglycemic/hypoxic condition (Fig. 1E). The sensitivities of the hepatoma-derived HepG2 cells, which express wild-type p53, were also examined. The IC_{50} of gemcitabine for HepG2 cells was 2.9 μM under the normoglycemic/normoxic condition, and more than 300 μM under the hypoglycemic/hypoxic condition (Fig. 1F).

Cell-cycle progression and gemcitabine uptake under various culture conditions. During cell proliferation, cells must prepare to double all their components. The restriction of nutrient and oxygen supply might greatly influence the cell-cycle progression, through complex mechanisms.[31] Gemcitabine is incorporated into the DNA to exert its cytotoxicity.[32,33] Therefore, the cell-cycle analysis was conducted under the hypoglycemic/hypoxic condition. Newly synthesized DNA was labeled with 5-ethynil-2′-deoxyuridine (EdU), and the DNA content was labeled with 7-aminoactinomycin D, followed by multicolor analysis by flow-cytometry. About 45% of the cells under the normoglycemic/normoxic condition and 41% of the cells under the hypoglycemic/hypoxic condition were in the S-phase. Thus, the S-phase population was almost the same under both conditions. Closer analysis of the S-phase populations under both conditions indicated that the numbers of cells in the late S and G2 phases were reduced under the hypoglycemic/hypoxic condition, indicating S-phase prolongation (Fig. 2A). The cellular uptake and DNA incorporation of gemcitabine were directly assessed using [^{3}H]-labeled gemcitabine. Cells were cultured under the normoglycemic/normoxic or hypoglycemic/hypoxic condition for 24 h, followed by incubation with 1 μM [^{3}H]-gemcitabine for 3 h. The cellular uptake of gemcitabine was almost

doi: 10.1111/j.1349-7006.2011.01880.x

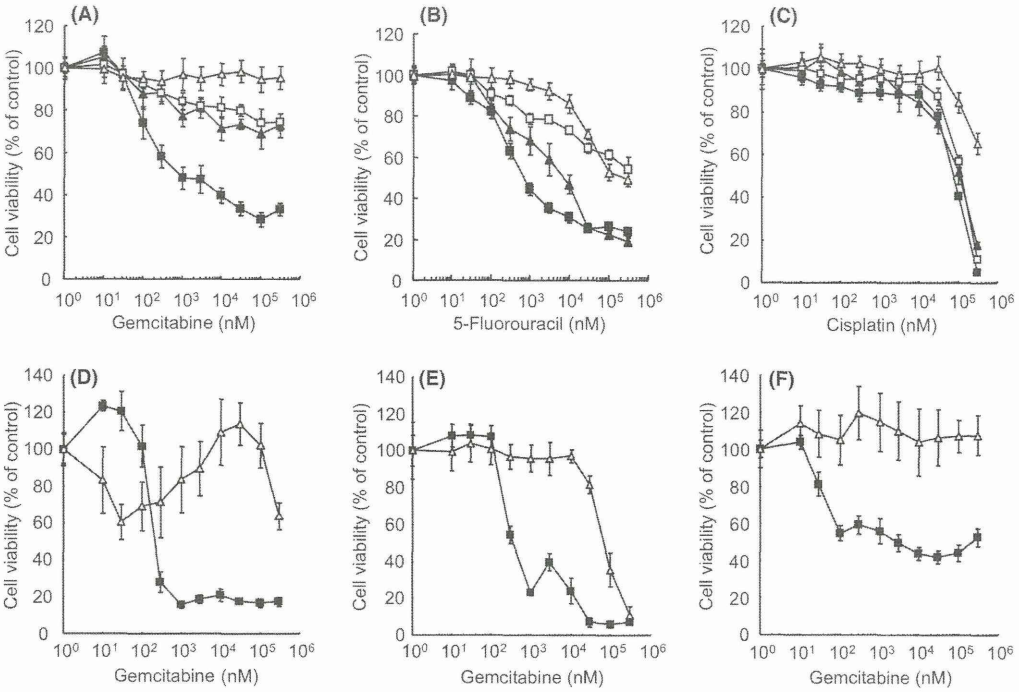

Fig. 1. Effect of the culture condition on the cytotoxicity of anticancer drugs. The cytotoxicity of (A) gemcitabine, (B) 5-fluorouracil and (C) cisplatin on the PANC-1 cells was examined. Cytotoxicity of gemcitabine on (D) the Capan-1, (E) PSN-1 and (F) HepG2 cells were also examined. (■) normoglycemic/normoxic, (▲) hypoglycemic/normoxic, (□) normoglycemic/hypoxic, and (△) hypoglycemic/hypoxic conditions.

twofold higher and the DNA incorporation of [^{3}H]-gemcitabine was almost fivefold higher under the hypoglycemic/hypoxic condition than under the normoglycemic/normoxic condition (Fig. 2B).

Gemcitabine-induced checkpoint activation and S-phase arrest. DNA incorporation of gemcitabine cause the replication fork to stall; this, in turn, induces S-phase checkpoint activation and S-phase arrest or apoptosis.[34,35] To analyze the signaling by gemcitabine-induced DNA lesions, we examined checkpoint kinase activations. After 12 h incubation in the presence or absence of 1 and 100 μM gemcitabine, phosphorylation of H2AX, Chk1 and Chk2 were induced by gemcitabine equally under different culture conditions (Fig. 2C). We further examined gemcitabine-induced S-phase arrest using propidium iodide staining and flow-cytometric analysis. S-phase arrest was equally induced by gemcitabine under the normoglycemic/normoxic and hypoglycemic/hypoxic conditions (Fig. 2D).

Effect of inhibition of Chk1 signaling on the cytotoxicity of gemcitabine. Previous studies have shown that UCN-01 and Gö6976 sensitized cells to gemcitabine via Chk1 inhibition, resulting in abrogation of the cell cycle arrest and subsequent cell death.[36–39] We examined the sensitivity of Chk1 signaling-inhibited cells to gemcitabine under the hypoglycemic/hypoxic condition. Western blot analysis showed that 1 μM of the Chk1 inhibitors, UCN-01 and Gö6976, reduced the phosphorylation of cdc25c, a downstream mediator of Chk1 (Fig. 3A); UCN-01 and Gö6976 lowered the IC_{50} of gemcitabine by more than 10 times under the normoglycemic/normoxic condition, but not under the hypoglycemic/hypoxic condition (Fig. 3B,C). Similar results were obtained with 10 μM UCN-01 or Gö6976. To confirm these results, the effect of an RNAi for Chk1 was examined. The RNAi effectively suppressed Chk1 activation under both the normoglycemic/normoxic and hypoglycemic/hypoxic conditions (Fig. 3D); however, Chk1 suppression enhanced the sensitivity of the cells to gemcitabine only under the normoglycemic/normoxic condition (Fig. 3E).

Effect of inhibition of the HIFs and PI3K/Akt signaling on the sensitivity of the cancer cells to gemcitabine. HIF-1α is induced by hypoxia and modifies cell survival.[40,41] Under the hypoxic condition, the HIF-1α protein levels increased rapidly to peak within 2 h and thereafter decreased (Fig. 4A). The HIF-2α protein level was also rapidly induced within 2 h, and maintained for 24 h. The HIF-1α protein level decreased, but not the HIF-2α protein levels, under the hypoglycemic condition (Fig. 4A). To evaluate the involvement of the HIFs in the resistance to gemcitabine, HIF-1α or HIF-2α expression was suppressed by RNAi and the sensitivity of the cells to gemcitabine was examined. RNAi for HIF-1α and HIF-2α effectively suppressed the hypoxia-induced accumulation of the respective proteins (Fig. 4B). Knockdown of HIF-1α, HIF-2α or HIF-1/2α did not have any effect on the sensitivity of the cells to gemcitabine under hypoxic condition (Fig. 4C–E). Akt is known to be activated by hypoglycemic condition and to play some roles in cell survival.[42,43] In our study, marked increase of Akt phosphorylation at ser473 was observed within 2 h under both the hypoglycemic and hypoxic condition, which was sustained for at least 24 h; the increase was, however, more evident under the hypoxic condition (Fig. 4A). To examine the involvement of PI3K/Akt signaling in the drug resistance, we utilized a PI3K inhibitor, LY294002. After treatment with LY294002 (10 and 20 μM) for 24 h, Akt phosphorylation was effectively inhibited to less than the basal level (Fig. 4F). Although treatment with 20 μM of LY294002 reduced the IC_{50} of gemcitabine by 15-fold under the normoglycemic/normoxic condition, it had little effect under the hypoglycemic/normoxic condition (Fig. 4G).

Effect of combined inhibition of Chk1 and HIF signaling on the drug resistance induced by hypoglycemic/hypoxic condition. Inhibition of either checkpoint to produce release from the gemcitabine-induced S-phase arrest or of cell-survival signaling under hypoxia, HIFs, and under hypoglycemia Akt, each alone was not effective to ameliorate the resistance to gemcitabine. We examined the combined inhibition of Chk1 and

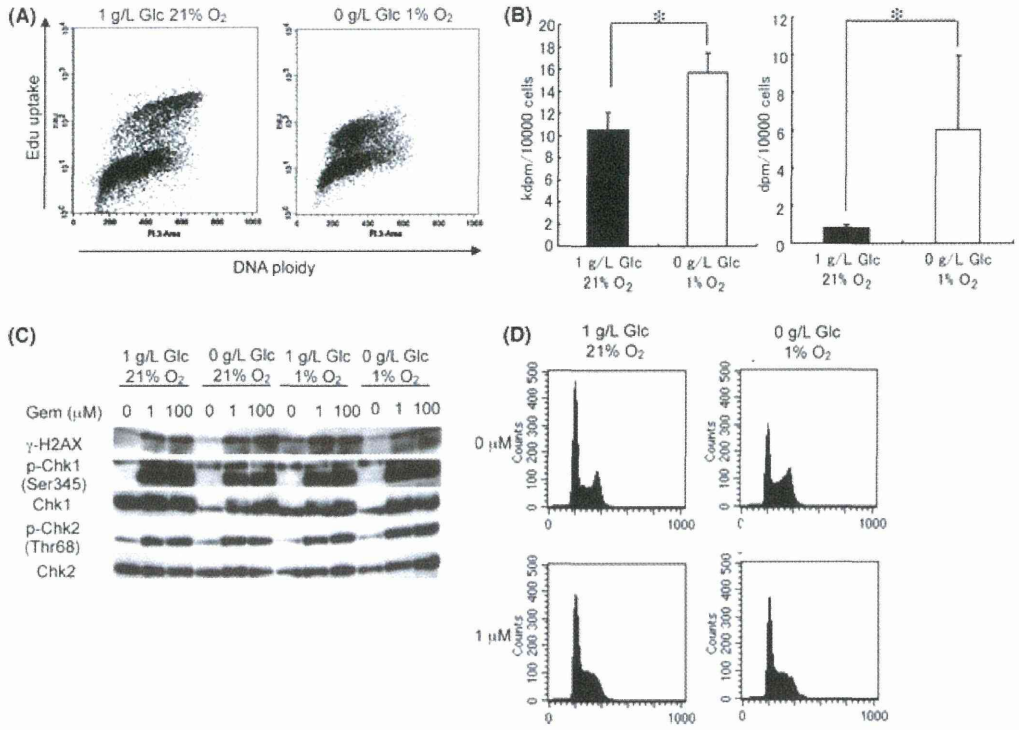

Fig. 2. Cell-cycle progression, uptake of gemcitabine and gemcitabine-induced cellular responses under various conditions. (A) Representative cell-cycle distribution detected by EdU incorporation and flow cytometry. Three independent experiments were carried out. (B) Cellular uptake and DNA incorporation of [^{3}H] gemcitabine (*$P < 0.05$). (C) Phosphorylations of H2AX, Chk1 and Chk2 detected by Western blot analysis after 12 h treatment with the indicated concentration of gemcitabine. (D) Representative DNA ploidy patterns after 24 h treatment with 1 μM gemcitabine. Three independent experiments were carried out.

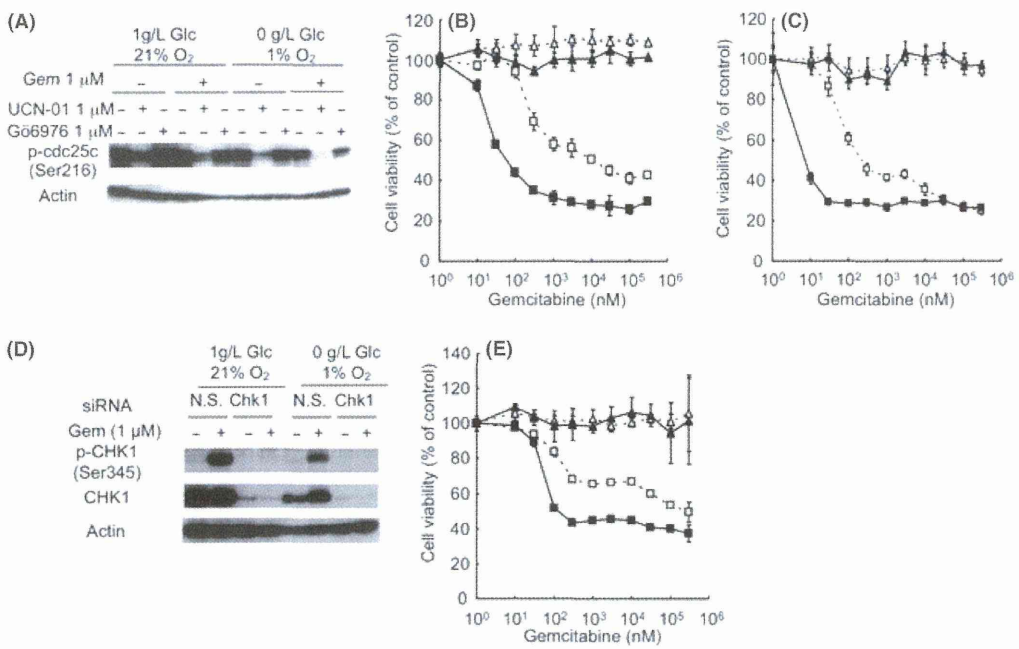

Fig. 3. Effect of inhibition of Chk1 signaling on the sensitivity of cells to gemcitabine. (A) Western blot analysis of cdc25c in the presence of Chk1 inhibitors under the indicated conditions. Cytotoxicity of gemcitabine in the presence or absence of 1 μM (B) UCN-01, (C) or Gö6976 under (■ or □) normoglycemic/normoxic condition or (▲ or △) hypoglycemic/normoxic condition. (D) Western blot analysis of Chk1 expression and activation. (E) The cytotoxicity of gemcitabine with or without Chk1 knockdown under (□ or ■) normoglycemic/normoxic condition or (△ or ▲) hypoglycemic/hypoxic condition.

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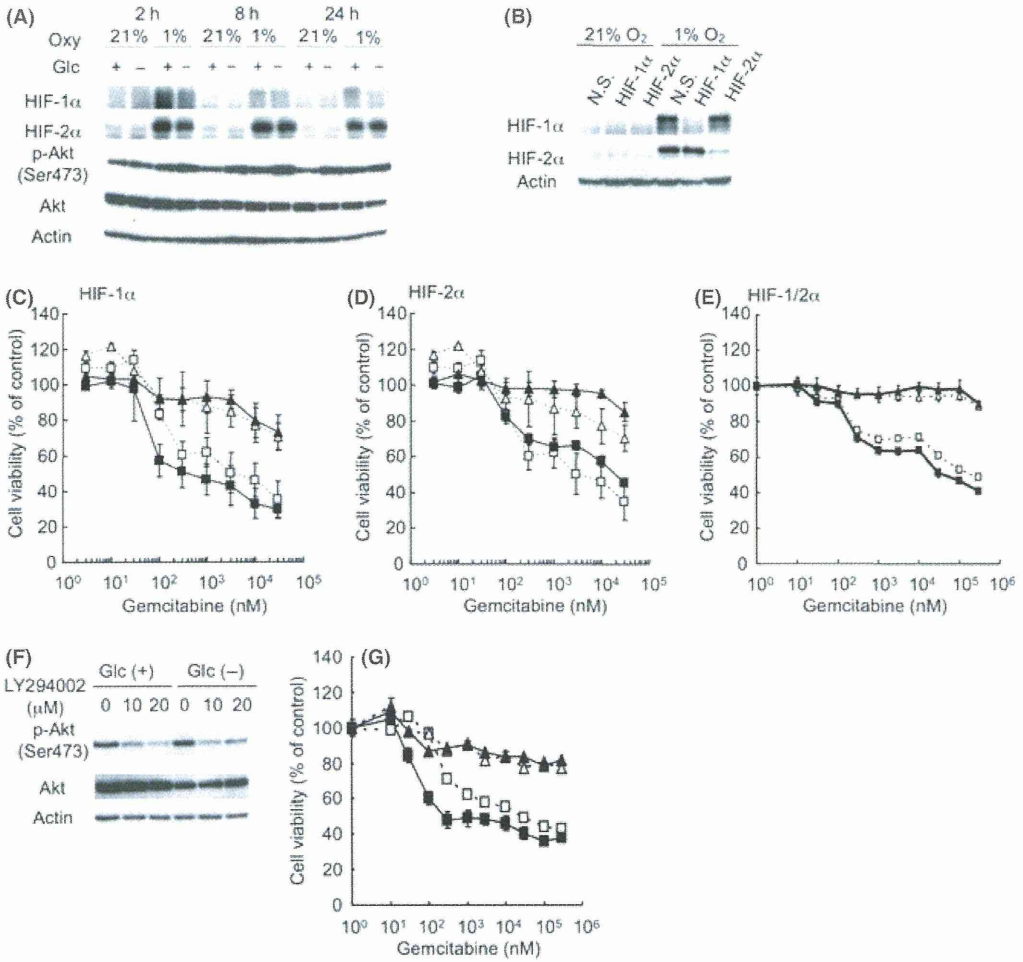

Fig. 4. Effects of inhibition of HIFs and PI3K/Akt signaling on the sensitivity of the cells to gemcitabine. (A) Western blot analysis for HIF1α and 2α accumulation and Akt phosphorylation under the indicated oxygen tension, normoxia (21%), or hypoxia (1%), and in the presence of a glucose concentration of 1 g/L (+) or 0 g/L (−). (B) Western blot analysis for HIF1α and 2α protein in cells treated with HIF-1α or HIF-2α siRNA. The cytotoxicity of gemcitabine on (C) HIF-1α, (D) HIF-2α or (E) HIF-1/2α knockdown cells or control cells under (■ or □) normoglycemic/normoxic condition or (▲ or △) normoglycemic/hypoxic condition. (F) Phosphorylation of Akt in the presence of 10 or 20 μM LY294002 under the indicated culture conditions. (G) Cytotoxicity of gemcitabine in the presence or absence of 20 μM LY294002 under (■ or □) normoglycemic/normoxic condition or (▲ or △) hypoglycemic/normoxic condition.

HIF signaling: HIF-1α, HIF-2α, or HIF-1/2α knockdown cells were examined for their sensitivity to gemcitabine in the presence of 1 μM UCN-01; however, even such combined inhibition was found to have no effect on the sensitivity of the cells to gemcitabine under the hypoxic condition (Fig. 5).

Effect of combined inhibition of Chk1 and PI3K signaling on the drug resistance induced by hypoglycemic/hypoxic condition. Combined inhibition of Chk1 and PI3K signaling was examined. As shown in Figure 6 1μM UCN-01 and 20 μM LY294002 strongly enhanced gemcitabine cytotoxicity under both normoglycemic/normoxic and hypoglycemic/hypoxic conditions, although the effect under the hypoglycemic/hypoxic condition was less pronounced (Fig. 6A). On the other hand, combined treatment with 1 μM Gö6976 and 20 μM LY294002 enhanced the sensitivity of the cells to gemcitabine only under the normoglycemic/normoxic condition (Fig. 6B). In order to confirm if the effect of UCN-01 was due to inhibition of Chk1 activation or inhibition of some other target, the effect of the RNAi on Chk1 activation was examined. Chk1 siRNA and 20 μM LY294002 enhanced the sensitivity of the cells to gemcitabine under the normoglycemic/normoxic condition; however, it had no any effect under the hypoglycemic/hypoxic condition.

Discussion

As clearly shown in the present work, hypoxia and hypoglycemia had a large impact on the cellular sensitivity to anticancer drugs in different cancer cell lines. In most cases, the mechanism underlying the drug resistance is regarded as decreased cellular drug uptake. Multidrug resistance is one of major cellular mechanisms of drug resistance to a broad spectrum of anticancer drugs, and this phenotype is associated with an increased drug efflux from the cells caused by overexpression of the ABC transporter. In the present work, hypoglycemic/hypoxic condition also induced multidrug resistance; however, our findings clearly indicated that there was no reduction of gemcitabine uptake and incorporation under the hypoglycemic/hypoxic condition. The S-phase population was similar under the normoglycemic/normoxic and hypoglycemic/hypoxic conditions, with accompanying S-phase prolongation. S-phase prolongation might be due to the depletion of *de novo* synthesis of nucleotides caused by

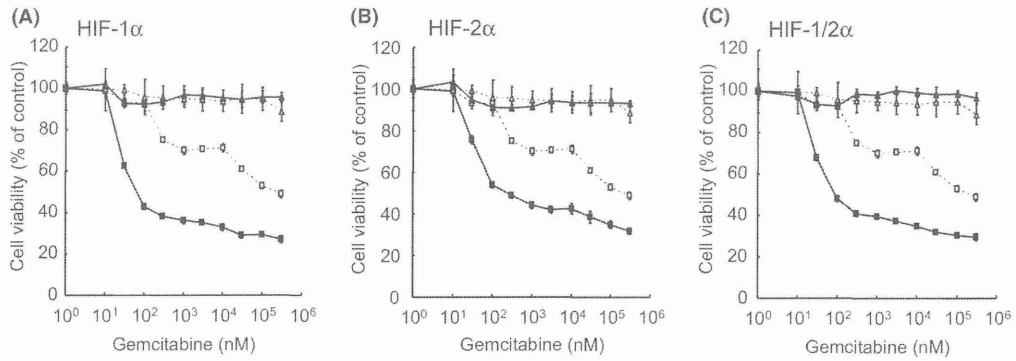

Fig. 5. Effect of combined inhibition of Chk1 and HIF signaling on the sensitivity of the cells to gemcitabine. Cells were treated with gemcitabine in the presence or absence of 1 μM UCN-01 plus RNAi for (A) HIF-1α, (B) HIF-2α or (C) HIF-1/2α under (■ or □) normoglycemic/normoxic condition or (▲ or △) normoglycemic/hypoxic condition.

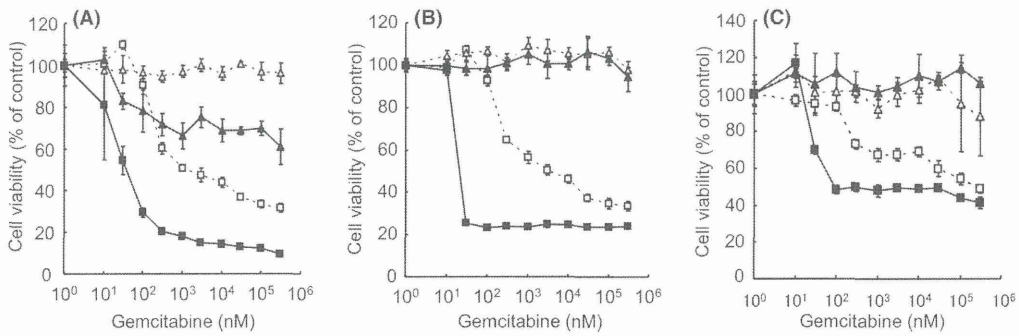

Fig. 6. Effect of combined inhibition of Chk1 and PI3K on the sensitivity of the cells to gemcitabine. Cells were treated with gemcitabine in the presence or absence of 1 μM (A) UCN-01, (B) 1 μM Gö6976 or (C) RNAi for Chk1, and 20 μM LY294002 under (■ or □) normoglycemic/normoxic condition or (▲ or △) hypoglycemic/hypoxic condition.

insufficiency of the pentose phosphate shunt supply. Nevertheless, it was not involved in DNA incorporation of gemcitabine under the hypoglycemic/hypoxic condition. Following its incorporation into DNA, gemcitabine blocks the extension of DNA and stall replication forks, leading to DNA damage. The DNA damage is recognized by sensor molecules that recruit and phosphorylate H2AX protein in the damaged DNA region.[44] Sensor molecules also phosphorylate checkpoint kinase causing its activation and arresting the cell cycle in the S phase.[45] The present study showed that phosphorylation of H2AX, Chk1 and Chk2 were induced by gemcitabine equally under the normoglycemic/normoxic and hypoglycemic/hypoxic conditions, leading to S-phase arrest. During checkpoint kinase activation and cell cycle arrest, phosphorylation of H2AX is known to be recruited by other DNA repair proteins, such as Mre11/Rad50/Nbs1, in the DNA damage region, resulting in activation of the DNA repair pathway.[46,47] Chronic hypoxia has been reported to suppress DNA repair protein activity.[48,49] The increased DNA incorporation of gemcitabine under the hypoglycemic/hypoxic condition may be caused by suppression of the DNA repair pathway.

Modulation of the cellular responses to DNA-damaging agents by checkpoint abrogators or inhibitors of cell survival signaling is an active area of research, since it has been believed that the interference of these signalings may enhance the therapeutic efficacy of anticancer drugs.[50] The S-phase checkpoint consists of a hierarchal regulatory cascade initiated by the activation of Chk1. In the present work, Chk1 inhibitors and Chk1 siRNA enhanced the cytotoxicity of gemcitabine under the normoglycemic/normoxic condition, consistent with other reports.[51–54] However, the abrogation of Chk1 activation did not affect the sensitivity of the cells to gemcitabine under the hypoglycemic/hypoxic condition. Tumor hypoxia has been well-studied, and previous reports have proposed that HIF-1α plays a critical role in determining cell survival and death,[40,41] while knockdown of HIF1α or HIF2α using siRNA did not affect the sensitivity of the cells to gemcitabine under the hypoxic condition in the present study. The PI3K/Akt pathway is well-known for its anti-apoptotic and cell survival activity under various conditions, including hypoxia and hypoglycemia,[55–57] but our results showed that the PI3K inhibitor LY294002 sensitized the cells to gemcitabine only under the normoglycemic/normoxic condition. We examined combined inhibition of Chk1 and of the cell survival pathways-sensitized cells to gemcitabine under the hypoglycemic/hypoxic condition. In the present work, the combination of UCN-01 and LY294002 partly abrogated the hypoglycemic/hypoxia-induced drug resistance, whereas the combination of Gö6976 or Chk1 siRNA with LY294002 had no such effect. These observations suggest that UCN-01 had a different target from Gö6976 in the mechanism of sensitizing the cells to gemcitabine under the hypoglycemic/hypoxic condition. UCN-01 has been reported to induce apoptosis in S-phase-arrested cells, not through Chk1 inhibition, although the precise mechanisms remain poorly understood.[58] We attempted to identify the kinase signaling responsible for the hypoglycemic/hypoxia-induced drug resistance in the targets of UCN-01; however, we did not obtain any clear results. PI3K and Akt are strongly expressed in some cancers, and have been found to be associated with a poor prognosis and increased tumor aggressiveness.[59,60] We previously reported that Akt

doi: 10.1111/j.1349-7006.2011.01880.x

expression was closely associated with cellular tolerance for nutrient deprivation.[61] The present work showed that Akt phosphorylation had a significant impact on the sensitivity of the PANC-1 cells to anticancer drugs.

In this study, we showed that hypoglycemic/hypoxic condition induced multidrug resistance. Combined kinase activations were involved in the hypoglycemic/hypoxia-induced drug resistance. Although the mechanism of cell death caused by gemcitabine is still unclear, the combined strategies described in the text might enhance the cytotoxicity of gemcitabine in clinical practice.

Acknowledgments

This work was supported by a Grant for the Third-Term Comprehensive 10-year Strategy for Cancer Control and 5-year Strategy for the Creation of Innovative Pharmaceuticals and Medical Devices from the Ministry of Health, Labour and Welfare, Japan.

Disclosure statement

No conflict of interest.

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doi: 10.1111/j.1349-7006.2011.01880.x

Biochemical and Biophysical Research Communications 392 (2010) 460–466

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 12 January 2010
Available online 18 January 2010

Keywords:
Efrapeptin F
Mitochondria
Glucose
Mitochondrial inhibitors
Nutrient deprivation
Glucose deprivation

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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doi:10.1016/j.bbrc.2010.01.050

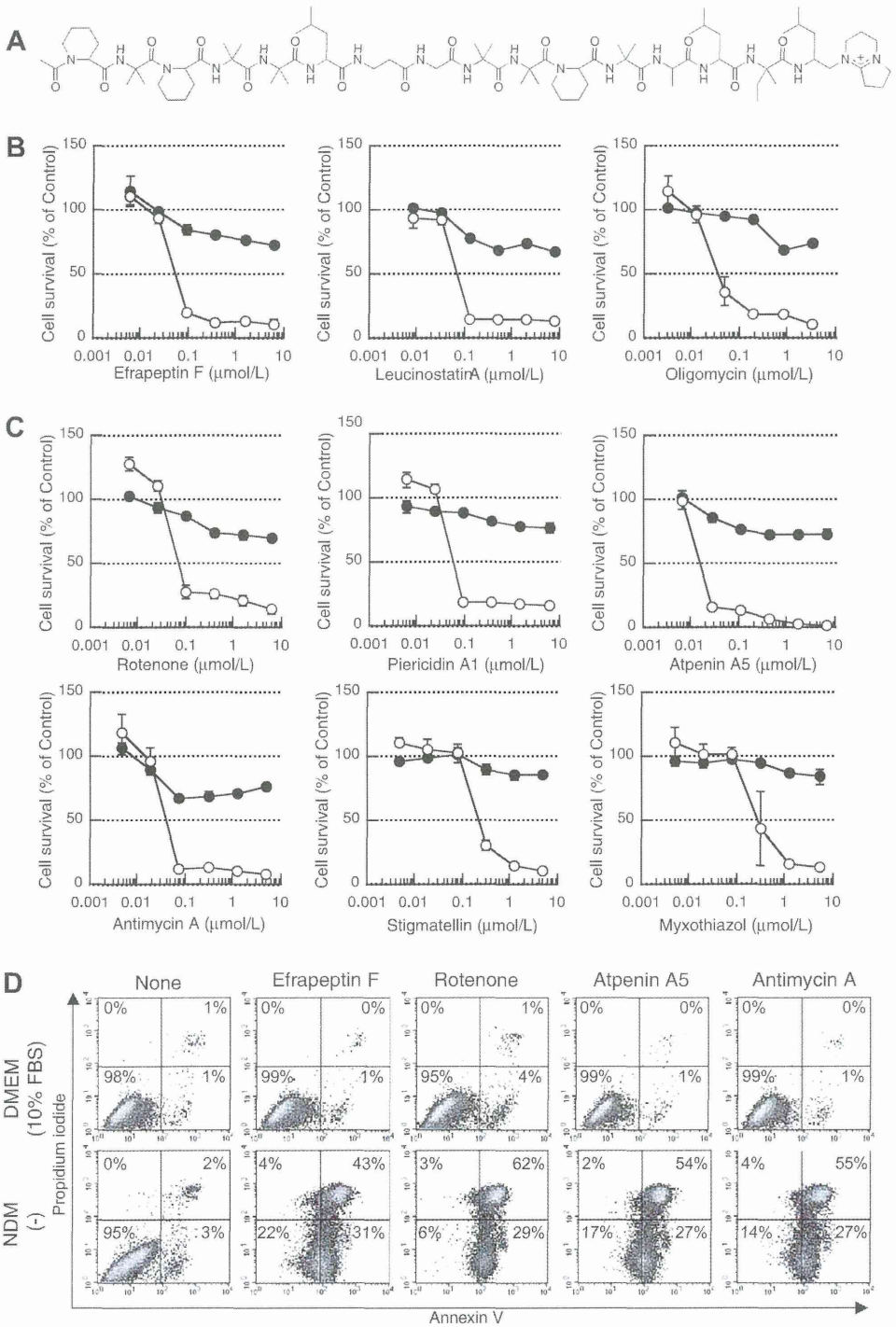
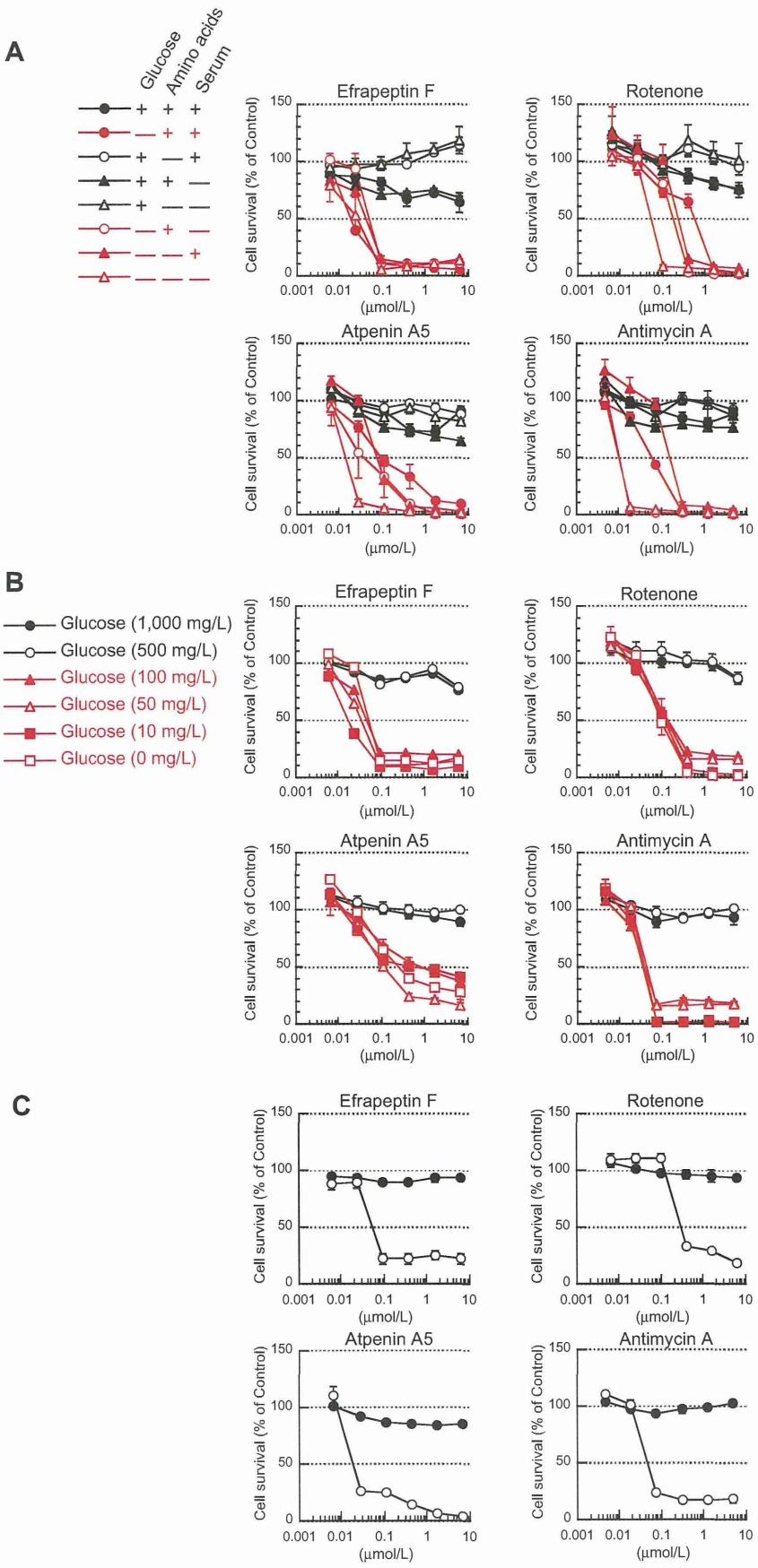


Fig. 1. Effect of efrapeptin F and mitochondrial inhibitors on PANC-1 survival under nutrient-deprived conditions. (A) Structure of efrapeptin F. (B) Effect of efrapeptin F and complex V inhibitors (leucinostatin A and oligomycin) on survival of PANC-1 cells in normal medium, DMEM (10% FBS) (●) and nutrient-deprived 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)
(μmo/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