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.

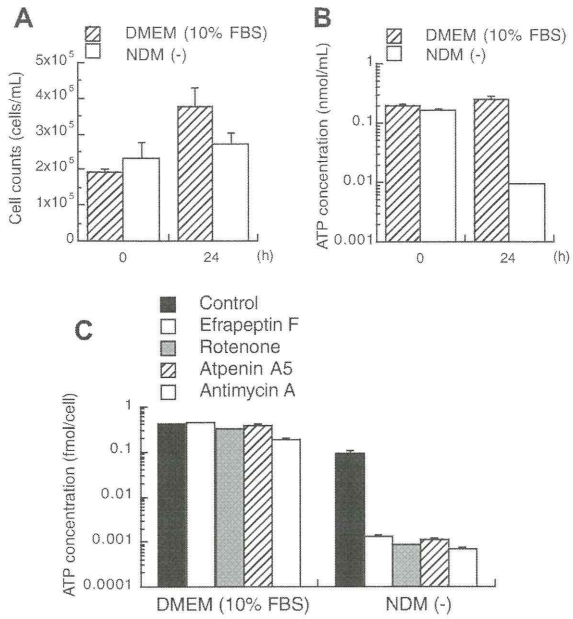

Fig. 3. Effect of mitochondrial inhibitors on cellular ATP levels of PANC-1 cells grown in nutrient-deprived medium. (A) Effect of nutrient starvation on PANC-1 cell growth. PANC-1 cells were incubated in DMEM (10% FBS) or NDM (−) for 24 h and cell numbers were measured by cell counting. (B) Cellular 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

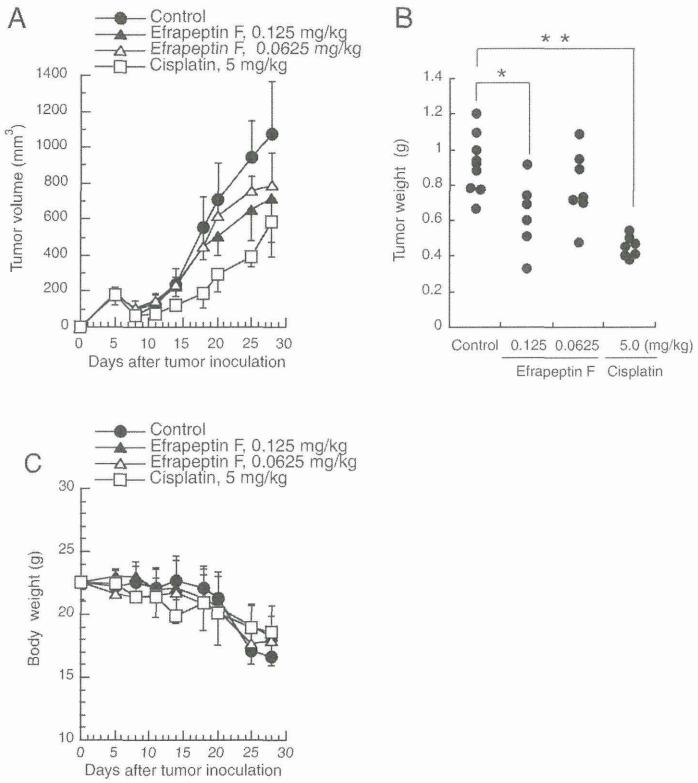

Fig. 4. Antitumor effect of efrapeptin F on PC-3 cells in SCID mice. PC-3 cells (1×10^7) were subcutaneously inoculated into SCID mice on day 0. Efrapeptin F was administrated intravenously twice weekly for 3 weeks from day 5. (A) Tumor volumes. *Y* axis, tumor volume (mm^3); *X* axis, time (day). (B) 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.

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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.

References

[1] P. Vaupel, F. Kallinowski, P. Okunieff, Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review, Cancer Res. 49 (1989) 6449–6465.
[2] J.M. Brown, A.J. Giaccia, The unique physiology of solid tumors: opportunities (and problems) for cancer therapy, Cancer Res. 58 (1998) 1408–1416.
[3] K. Izuishi, K. Kato, T. Ogura, T. Kinoshita, H. Esumi, Remarkable tolerance of tumor cells to nutrient deprivation: possible new biochemical target for cancer therapy, Cancer Res. 60 (2000) 6201–6207.
[4] R. Baserga, The contradictions of the insulin-like growth factor 1 receptor, Oncogene 19 (2000) 5574–5581.
[5] M.N. Pollak, E.S. Schernhammer, S.E. Hankinson, Insulin-like growth factors and neoplasia, Nat. Rev. Cancer 4 (2004) 505–518.
[6] S. Kunimoto, J. Lu, H. Esumi, Y. Yamazaki, N. Kinoshita, Y. Honma, M. Hamada, M. Ohsono, M. Ishizuka, T. Takeuchi, Kigamicins, novel antitumor antibiotics. I. Taxonomy, isolation, physico-chemical properties and biological activities, J. Antibiot. 56 (2003) 1004–1011.
[7] S. Kunimoto, T. Someno, Y. Yamazaki, J. Lu, H. Esumi, H. Naganawa, Kigamicins, novel antitumor antibiotics. II. Structure determination, J. Antibiot. 56 (2003) 1007–1012.
[8] J. Lu, S. Kunimoto, Y. Yamazaki, M. Kaminishi, H. Esumi, H. Esumi, Kigamicin D, a novel anticancer agent based on a new anti-austerity strategy targeting cancer cells' tolerance to nutrient starvation, Cancer Sci. 95 (2004) 547–552.
[9] I. Momose, S. Kunimoto, M. Osono, D. Ikeda, Inhibitors of insulin-like growth factor-1 receptor tyrosine kinase are preferentially cytotoxic to nutrient-deprived pancreatic cancer cells, Biochem. Biophys. Res. Commun. 380 (2009) 171–176.
[10] N.C. Denko, Hypoxia, HIF1 and glucose metabolism in the solid tumour, Nat. Rev. Cancer 8 (2008) 705–723.
[11] R.A. Gatenby, R.J. Gillies, Why do cancers have high aerobic glycolysis?, Nat Rev. Cancer 4 (2004) 891–899.
[12] G. Kroemer, J. Pouyssegur, Tumor cell metabolism: cancer's Achilles' heel, Cancer Cell 13 (2008) 472–482.
[13] A. Hirayama, K. Kami, M. Sugimoto, M. Sugawara, N. Toki, H. Onozuka, T. Kinoshita, N. Saito, A. Ochiai, M. Tomita, H. Esumi, T. Soga, Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry, Cancer Res. 69 (2009) 4918–4925.
[14] B. Levine, D.J. Klionsky, Development by self-digestion: molecular mechanisms and biological functions of autophagy, Dev. Cell 6 (2004) 463–477.
[15] S. Gupta, B.S. Krasnoff, W.D. Roberts, A.A.J. Renwick, S.L. Brinen, J. Clardy, Structures of the efrapeptins: potent inhibitors of mitochondrial ATPase from the fungus *Tolypocladium niveum*, J. Am. Chem. Soc. 113 (1991) 707–709.
[16] S. Gupta, B.S. Krasnoff, W.D. Roberts, A.A.J. Renwick, S.L. Brinen, J. Clardy, Structure of efrapeptins from the fungus *Tolypocladium niveum*: peptide inhibitors of mitochondrial ATPase, J. Org. Chem. 57 (1992) 2306–2313.
[17] R.L. Cross, W.E. Kohlbrenner, The mode of inhibition of oxidative phosphorylation by efrapeptin (A23871). Evidence for an alternating site mechanism for ATP synthesis, J. Biol. Chem. 253 (1978) 4865–4873.
[18] M. Kawada, I. Momose, T. Someno, G. Tsujiuchi, D. Ikeda, New atpenins, NBRI23477A and B, inhibit the growth of human prostate cancer cells, J. Antibiot. 62 (2009) 243–246.
[19] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[20] M. Ueki, K. Machida, M. Takeuchi, Antifungal inhibitors of mitochondrial respiration: discovery and prospects for development, Curr. Opin. Investig. Drugs 2 (2000) 387–398.
[21] N. Orme-Johnson, Direct and indirect inhibitors of mitochondrial ATP synthesis, Methods Cell Biol. 80 (2007) 813–826.
[22] H. Miyadera, K. Shiomi, H. Ui, Y. Yamaguchi, R. Masuma, H. Tomoda, H. Miyoshi, A. Osanai, K. Kita, S. Omura, Atpenins, potent and specific inhibitors of mitochondrial complex II (succinate-ubiquinone oxidoreductase), Proc. Natl. Acad. Sci. USA 100 (2003) 473–477.
[23] S. Dan, T. Tsunoda, O. Kitahara, R. Yanagawa, H. Zembutsu, T. Katagiri, K. Yamazaki, Y. Nakamura, T. Yamori, An integrated database of chemosensitivity to 55 anticancer drugs and gene expression profiles of 39 human cancer cell lines, Cancer Res. 62 (2002) 1139–1147.
[24] T. Yamori, Panel of human cancer cell lines provides valuable database for drug discovery and bioinformatics, Cancer Chemother. Pharmacol. 52 (Suppl. 1) (2003) S74–S79.
[25] T. Yamori, A. Matsunaga, S. Sato, K. Yamazaki, A. Komi, K. Ishizu, I. Mita, H. Edatsugi, Y. Matsuba, K. Takezawa, O. Nakanishi, H. Kohno, Y. Nakajima, H. Komatsu, T. Andoh, T. Tsuruo, Potent antitumor activity of MS-247, a novel DNA minor groove binder, evaluated by an in vitro and in vivo human cancer cell line panel, Cancer Res. 59 (1999) 4042–4049.
[26] C.V. Dang, G.L. Semenza, Oncogenic alterations of metabolism, Trends Biochem. Sci. 24 (1999) 68–72.
[27] R.M. Southerland, Cell and environment interactions in tumor microregions: the multicell spheroid model, Science 240 (1988) 178–184.
[28] G. Helmlinger, F. Yuan, M. Dellian, R.K. Jain, Interstitial pH and pO_2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation, Nat. Med. 3 (1997) 177–182.
[29] O. Warburg, On respiratory impairment in cancer cells, Science 124 (1956) 269–270.

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Leucinostatin A inhibits prostate cancer growth through reduction of insulin-like growth factor-I expression in prostate stromal cells

Manabu Kawada, Hiroyuki Inoue, Shun-Ichi Ohba, Tohru Masuda, Isao Momose and Daishiro Ikeda

Numazu Bio-Medical Research Institute, Microbial Chemistry Research Center, 18-24 Miyamoto, Numazu-shi, Shizuoka 410-0301, Japan

Fast Track

Targeting stroma in tumor tissues is an attractive new strategy for cancer treatment. We developed *in vitro* coculture system, in which the growth of human prostate cancer DU-145 cells is stimulated by prostate stromal cells (PrSC) through insulin-like growth factor I (IGF-I). Using this system, we have been searching for small molecules that inhibit tumor growth through modulation of tumor-stromal cell interactions. As a result, we have found that leucinostatins and atpenins, natural antifungal antibiotics, inhibit the growth of DU-145 cells cocultured with PrSC more strongly than that of DU-145 cells alone. In this study we examined the antitumor effects of these small molecules *in vitro* and *in vivo*. When DU-145 cells were coinoculated with PrSC subcutaneously in nude mice, leucinostatin A was found to significantly suppress the tumor growth more than atpenin B. The antitumor effect of leucinostatin A *in vivo* was not obtained against the tumors of DU-145 cells alone. RT-PCR experiments revealed that leucinostatin A specifically inhibited IGF-I expression in PrSC without effect on expressions of other IGF axis molecules. Leucinostatins and atpenins are known to abrogate mitochondrial functions. However, when we used mitochondrial DNA-depleted, pseudo-ρ^0 cells, we found that one of leucinostain A actions certainly depended on mitochondrial function, but it actually inhibited the growth of DU-145 cells more strongly in coculture with pseudo-ρ^0 PrSC and reduced IGF-I expression in pseudo-ρ^0 PrSC. Taken together, our results suggested that leucinostatin A inhibited prostate cancer cell growth through reduction of IGF-I expression in PrSC.

Growing evidence indicates that the stroma plays a critical role in the growth and metastasis of various cancers, including colorectal,[1] breast,[2,3] pancreatic[4] and prostate cancer.[5] The constituents of stroma vary in each tissue, but they generally include fibroblasts, macrophages, endothelial cells and extracellular matrix.[6,7] Among these components, certain types of fibroblasts appear to enhance tumor growth and others suppress it.[2,8–10] Fibroblasts that enhance tumor growth are especially referred to as cancer-associated fibroblasts or activated fibroblasts,[11,12] and have distinct characters from normal fibroblasts as they express both vimentin and smooth muscle α-actin (SM α-actin), indicating a myofibroblast phenotype.[6] Theses cells secrete various factors favorable for tumor cell growth, such as growth factors, cytokines and adhesion molecules.[6,12] Thus, tumor-stromal cell interactions can promote tumor growth and metastasis through secreted factors and cell-cell adhesion.[9,10,12,13] Although various kinds of growth factors and cytokines are reported to be involved in tumor-stromal cell interactions, many studies suggest that insulin-like growth factor I (IGF-I) plays an important role in prostate tumor development.[14,15]

The fact that stromal cells can regulate tumor development positively or negatively drives us to consider the modulation of tumor-stromal cell interactions could be an attractive new strategy for the treatment of cancer.[16,17] Some growth factors and antibodies actually suppress the growth of some cancers.[18–20] However, small molecules that modulate tumor-stromal cell interactions are less reported. We therefore constructed an *in vitro* coculture system of prostate cancer cells and PrSC, designed to mimic characteristics of tumors *in vivo*.[14,21] Using this system, we have been searching for small-molecule modulators of tumor-stromal cell interactions. As a result, we found that phthoxazolin A inhibits its growth of prostate cancer cells through reduction of IGF-I secretion from PrSC by suppressing myofibroblast differentiation of PrSC.[22]

Moreover, we have recently found that atpenins along with new congeners inhibit growth of prostate cancer cells possibly through modulation of tumor-stromal cell interactions. Further screening has brought about the finding that leucinostatins, fungal metabolites, have great activities in our

Key words: prostate cancer, prostate stroma, natural compound, IGF-I, tumor growth

Additional Supporting Information may be found in the online version of this article.

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Correspondence to: Manabu Kawada, Numazu Bio-Medical Research Institute, Microbial Chemistry Research Center, 18-24 Miyamoto, Numazu-shi, Shizuoka 410-0301, Japan, Fax: +81-55-922-6888, E-mail: kawadam@bikaken.or.jp

assay system. In this study, we examined the effects of leucinostatins and atpenins *in vitro* and *in vivo* in respect to modulation of tumor-stromal cell interactions. Here we report that leucinostatin A abrogates tumor-stromal cell interactions through inhibition of IGF-I expression in PrSC and inhibits the growth of prostate cancer cells *in vitro* and *in vivo*.

Material and Methods

Reagents

Rhodanile blue was purchased from Aldrich (Milwaukee, WI). Insulin, hydrocortisone, rotenone, and antimycin A1 were obtained from Sigma (St. Louis, MO). Transferrin was obtained from Wako Pure Chemical Industries (Tokyo, Japan). Recombinant human basic fibroblast growth factor (bFGF) was purchased from Pepro Tech (London, United Kingdom). Antibodies used were anti-IGF-IRβ (sc-713) (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phosphotyrosine (05-321) (Upstate Biotechnology, Lake Placid, NY). Leucinostatin A and B and atpenin A5 and B were purified from microbial cultured broth in our laboratory.[23]

Cells

The human prostate cancer cell line DU-145 was obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH), 100 U/ml penicillin G and 100 μg/ml streptomycin at 37°C with 5% CO_2. The human normal prostate stromal cells (PrSC) were obtained from Bio Whittaker (Walkersville, MD) and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, ITH (5 μg/ml insulin, 5 μg/ml transferrin, and 1.4 μM hydrocortisone), and 5 ng/ml bFGF at 37°C with 5% CO_2.

Coculture experiments

A microplate assay method for the selective measurement of epithelial tumor cells in coculture with stromal cells using rhodanile blue dye was performed as previously described.[21] PrSC were first inoculated into 96-well plates at 5,000 cells per well in 100 μl DMEM supplemented with ITH and 0.1% FBS in the presence of various concentrations of test compounds. After 2 days, 10 μl aliquots of DU-145 cell suspension (5,000 cells) in serum-free DMEM were inoculated onto monolayers of PrSC, and the cells were cultured for further 3 days. For monoculture of DU-145 cells, assay medium alone was first incubated in the presence of test compounds for 2 days at 37°C, DU-145 cells were then inoculated as described above, and cultured for further 3 days.

Antitumor effect in vivo

Male or female nude mice, 6-weeks old, were purchased from Charles River Breeding Laboratories (Yokohama, Japan) and maintained in a specific pathogen-free barrier facility according to our institutional guidelines. DU-145 cells (8×10^6) were trypsinized and resuspended with or without PrSC (8×10^6) in 0.3 ml of 10% FBS-DMEM and then combined with 0.5 ml of growth factor-reduced Matrigel (BD Biosciences). One hundred microliters of the cell suspension (1×10^6 cells) were injected subcutaneously in the left lateral flank of mice. Five mice were used for each experimental set. Tumor volume was estimated using the following formula: tumor volume (mm^3) = (length × $width^2$)/2. After the indicated times, tumors were surgically dissected.

Reverse transcription-PCR and real time PCR analyses

PrSC (2.5×10^5) or DU-145 cells (2.5×10^5) were cultured in DMEM supplemented with ITH and 0.1% FBS in the presence of various concentrations of test compounds for the indicated times. Total RNA was isolated using the RNeasy Minikit (Qiagen, Hilden, Germany). cDNAs were synthesized using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) from 1 μg aliquots of RNA and amplified using *Taq* DNA polymerase (Promega). The specific primers used were: cytochrome b (334-bp), 5′-GGCTTACTTCTCTT CATTCTCTCCT-3′ (sense) and 5′- GGTTGTCCTCCAATT CATGTTA-3′ (antisense)[24]; and other primers as reported elsewhere.[14,22] PCR were optimized for each set of primers using different numbers of cycles to ensure that amplification occurred in a linear range. After amplification, products were electrophoresed in 2% agarose gels, stained with SYBR Green I (Cambrex Bio Science, Rockland, ME) and analyzed with a FLA-5000 image analyzer (Fujifilm, Tokyo, Japan). For real time PCR, gene expression was measured in a Thermal Cycler Dice Real Time System (Takara, Shiga, Japan) with SYBR Premix Ex Taq II (Takara).

Preparation of cell lysates and Western blotting

For preparation of PrSC-CM, PrSC (3×10^5) were cultured in DMEM supplemented with 0.1% dialyzed FBS in the presence of various concentrations of test compounds for 2 days. DU-145 cells (5×10^5) were cultured in serum-free DMEM overnight and treated with PrSC-CM for 30 min at 37°C. Cell lysates were prepared and applied to Western blotting with antibodies as previously described.[25]

Preparation of Pseudo-ρ^0 cells

According to the method of Tartier *et al.*,[26] PrSC or DU-145 cells were cultured for 7 days in the presence of 250 ng/ml ethidium bromide in DMEM supplemented with 4.5 mg/ml glucose, 50 μg/ml uridine and 2 mM pyruvate to compensate for the respiratory metabolism deficit.

Statistical analysis

All data are representative of 2 or 3 independent experiments with similar results. Statistical analysis was carried out using Student's *t*-test.

Results

Leucinostatins and atpenins strongly inhibited growth of DU-145 cells in coculture with PrSC

We have been screening for modulators of tumor-stromal cell interactions of prostate cancer cells among natural compounds in microbial cultured broths. We have recently found that atpenins, which were originally reported as antifungal antibiotics and specific inhibitors of mitochondrial complex II,[27–29] inhibited the growth of DU-145 cells in coculture with PrSC more strongly than that of DU-145 cells cultured alone (monoculture) (Fig. 1*b*).[23] Our further screening resulted in the finding of another active microbial cultured broth. We purified the active components and identified them as leucinostatin A and B (Fig. 1*a*). Leucinostatins were originally discovered as antifungal antibiotics.[30–32] As shown in Figure 1*a*, leucinostatins marginally inhibited the growth of DU-145 cells in monoculture, but they strongly inhibited that of DU-145 cells when the cells were cocultured with PrSC. Leucinostatin A did not affect the growth of PrSC (Fig. 1*c*) and leucinostatin B as well as atpenins did not show any cytotoxic effects against PrSC under microscopic observation (data not shown). Rhodanile blue that preferentially stained epithelial cells showed selective decrease in cell numbers of DU-145 cells by leucinostatin A in the coculture with PrSC compared with DU-145 cells alone (Fig. 1*d*). On the other hand, leucinostatin A equally inhibited the growth of NCI-H460 human lung cancer cells in the presence or absence of human lung fibroblasts (Supporting Information Fig. 1). It is suggested that leucinostatin A selectively acts on a particular interaction between tumor and stromal cells.

Leucinostatin A suppressed growth of DU-145 tumors in vivo

Since we obtained rather larger amounts of leucinostatin A and atpenin B compared with the respective analogues, we evaluated the antitumor effects of leucinostatin A and atpenin B *in vivo*. To reflect the result *in vitro*, we inoculated DU-145 cells along with PrSC subcutaneously in nude mice. As a result, leucinostatin A was found to suppress the growth of DU-145 tumors more significantly than atpenin B at lower doses (Fig. 2*a*). Since antitumor effect of leucinostatin A was more potent than atpenin B, we used leucinostatin A mainly for further studies.

Antitumor effect of leucinostatin A depended on coinoculation of PrSC

Because leucinostatin A inhibited the growth of DU-145 cells in coculture with PrSC more strongly than that in monoculture (Fig. 1), we next examined whether the antitumor effect of leucinostatin A depends on the presence of PrSC *in vivo*. As reported previously, coinoculation of PrSC increased the growth of DU-145 tumors (Fig. 2*b*).[14] Leucinostatin A suppressed the growth of DU-145 tumors when the cells were inoculated with PrSC, but it did not affect the growth of DU-145 tumors without PrSC (Fig. 2*b*). This result indicated that leucinostatin A virtually inhibited the additive growth of DU-145 tumors stimulated by coinoculation of PrSC.

Leucinostatin A inhibited IGF-I expression in PrSC

Growing evidence suggests that IGF-I plays a critical role in the development of prostate cancer.[14,15] As we previously reported,[14,33] PrSC are mixtures of fibroblasts and myofibroblasts and IGF-I secreted from PrSC enhances growth of DU-145 cells in the coculture with PrSC (Supporting Information Fig. 2). Furthermore, we have recently reported that phthoxazolin A, another small molecule modulator of tumor-stromal cell interactions, inhibited IGF-I expression in PrSC through suppression of myofibroblast differentiation of PrSC.[22] We therefore examined the effect of leucinostatin A on IGF axis molecules in PrSC. As a result, leucinostatin A was found to specifically inhibit IGF-I expression in PrSC among various IGF axis molecules (Fig. 3*a*). Real time RT-PCR analysis also confirmed that leucinostatin A reduced IGF-I mRNA in PrSC (Fig. 3*b*). Phthoxazolin A inhibits expressions of IGF-1, several IGFBPs, and SM α-actin, a marker of myofibroblast differentiation.[22] However, leucinostatin A failed to inhibit SM α-actin expression and it only slightly inhibited IGFBP-3 expression (Fig. 3*a*).

It is reported that DU-145 cells respond to IGF-I mitogenic signal through IGF-IR.[14] As shown in Figure 3*c* conditioned medium of PrSC increased the phosphorylation of IGF-IR in DU-145 cells due to secreted IGF-I in the conditioned medium.[22] In contrast, conditioned medium prepared from leucinostatin A-treated PrSC actually failed to increase the phosphorylation of IGF-IR in DU-145 cells, indicating the decreased amounts of IGF-I in the conditioned medium of PrSC. Furthermore, leucinostatin A-augmented inhibition of DU-145 cell growth in coculture with PrSC was partially recovered by the external addition of IGF-I (Supporting Information Fig. 3).

Action of leucinostatin A on coculture of DU-145 cells and PrSC was irrespective of mitochondrial function

It is reported that leucinostatins inhibit oxidative phosphorylation in mitochondria.[34] Atpenins are known to be specific inhibitors of mitochondrial complex II.[29] Therefore, we next examined whether the action of leucinostatin A depends on mitochondrial function in cells. We use pseudo-ρ^0 cells that lacked mitochondrial DNA.[26] To verify the status of mitochondrial DNA depletion, we examined the expression of cytochrome b, a component of complex III, which is encoded by mitochondrial DNA.[24] Exposure to ethidium bromide for 7 days resulted in the great reduction of cytochrome b expression in PrSC and DU-145 cells (Fig. 4*a*). Thus, we referred to these cells as pseudo-ρ^0 cells. Among IGF axis molecules IGFBP-3 expression was significantly decreased in pseudo-ρ^0 PrSC. SM α-actin expression was also reduced in

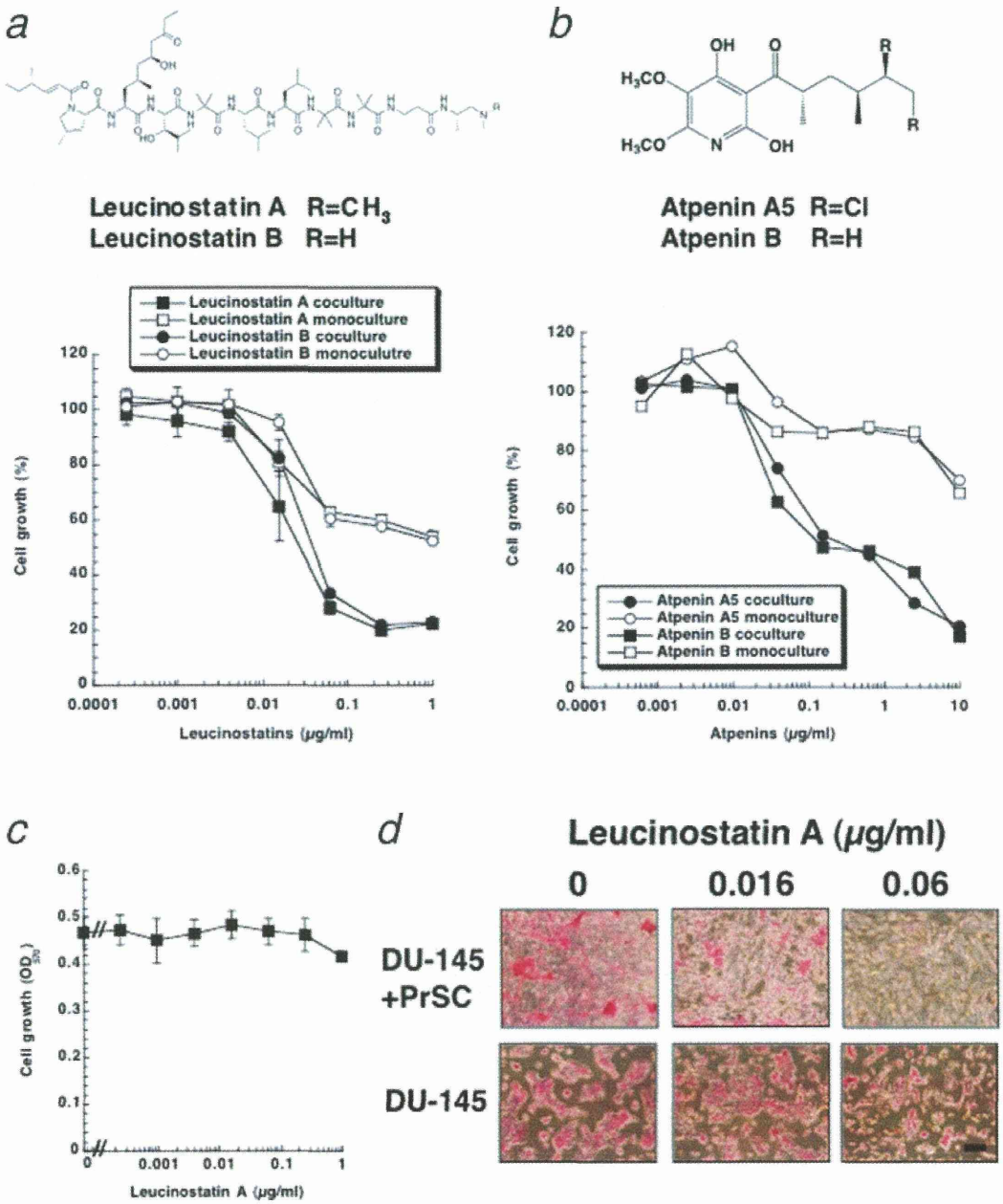

Figure 1. Effect of leucinostatins and atpenins on coculture of DU-145 cells and PrSC. (*a*) Effects of leucinostatin A (squares) and leucinostatin B (circles) on the growth of DU-145 cells alone (open plots) and DU-145 cells cocultured with PrSC (closed plots) were determined using rhodanile blue staining. Values are means ± SD of triplicate determinations. (*b*) Effects of atpenin A5 (circles) and atpenin B (squares) on the growth of DU-145 cells alone (open plots) and DU-145 cells cocultured with PrSC (closed plots) were determined using rhodanile blue staining. Values are means of duplicate determinations [standard errors (SE) less than 10%]. (*c*) Effect of leucinostatin A on the growth of PrSC was determined using MTT. PrSC were cultured alone with the indicated concentrations of leucinostatin A for 2 days. Values are means ± SD of triplicate determinations. (*d*) Representative photos of DU-145 cells cocultured with PrSC and DU-145 cells cultured alone after staining with rhodanile blue. Cells were treated with the indicated concentrations of leucinostatin A. Bar, 200 μm.

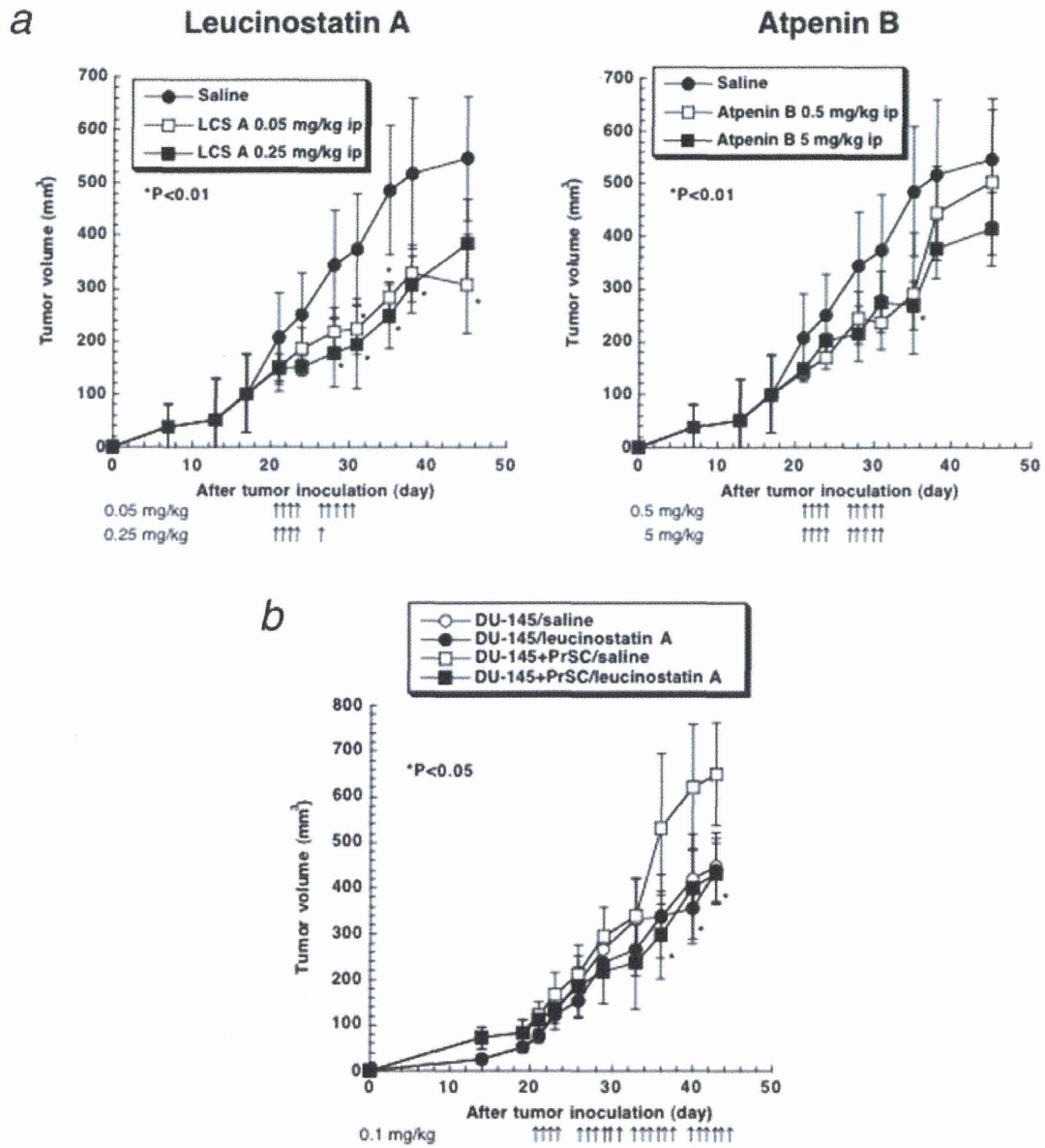

Figure 2. Effects of leucinostatin A and atpenin B on tumor growth of DU-145 cells *in vivo*. (*a*) DU-145 cells were inoculated subcutaneously with PrSC in female nude mice. Leucinostatin A or atpenin B was administered intraperitoneally at the indicated days (arrows). The values are means ± SD of 5 mice. *$p < 0.01$ versus the values with saline. (*b*) DU-145 cells were inoculated subcutaneously with (squares) or without PrSC (circles) in male nude mice. Leucinostatin A at 0.1 mg/kg was administered intraperitoneally at the indicated days (arrows). The values are means ± SD of 5 mice. *$p < 0.05$ versus the values with saline.

pseudo-ρ^0 PrSC suggesting suppression of myofibroblast differentiation, but IGF-I expression was only slightly decreased. Expressions of IGF axis molecules were not affected in pseudo-ρ^0 DU-145 cells. Using these pseudo-ρ^0 cells, we examined the effect of leucinostatin A on coculture of DU-145 cells and PrSC. As a result, leucinostatin A also inhibited the growth of DU-145 cells even cocultured with pseudo-ρ^0 PrSC more strongly than that in monoculture (Fig. 4*c*). In contrast, the growth inhibitory effect of leucinostatin A significantly weakened against pseudo-ρ^0 DU-145 cells suggesting that part of leucinostatin action actually depends on mitochondrial function in DU-145 cells. However, leucinostatin A apparently inhibited the growth of pseudo-ρ^0 DU-145 cells in coculture with PrSC more strongly than that in

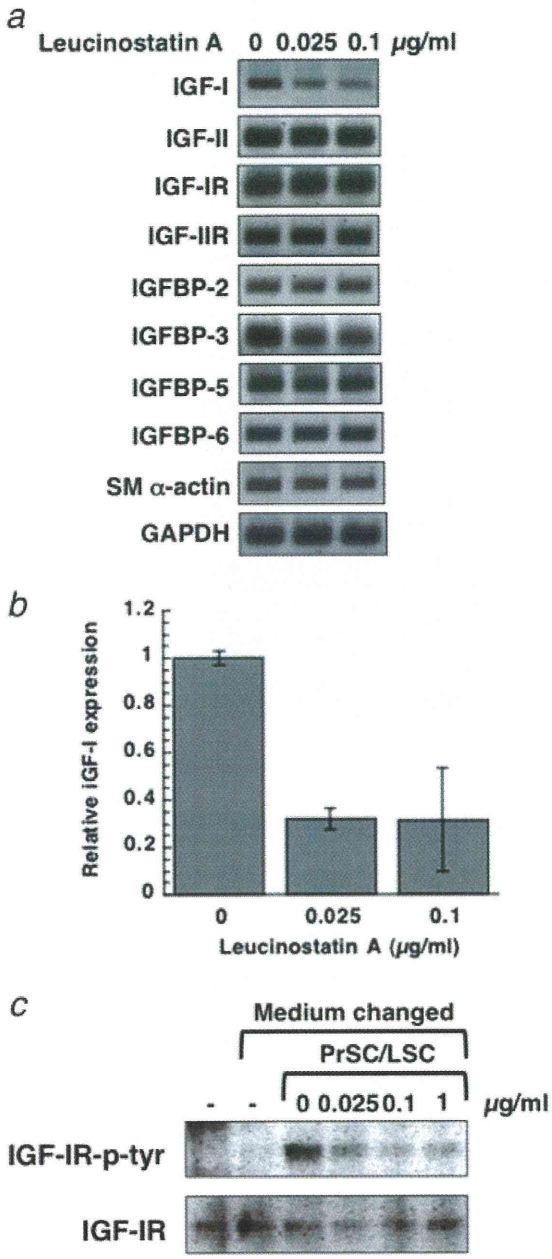

Figure 3. Effect of leucinostatin A on expression of IGF axis molecules in PrSC. (*a*) PrSC were cultured with leucinostatin A for 2 days. Total RNAs were collected and RT-PCR for the indicated molecules was carried out using specific primers. (*b*) IGF-I mRNA levels were analyzed by real time RT-PCR using GAPDH as a reference. (*c*) Conditioned medium was prepared from PrSC cultured with the indicated concentrations of leucinostatin A for 2 days (PrSC/LSC). DU-145 cells were treated with the conditioned medium or normal medium for 30 min (Medium changed). Cell lysates were prepared and applied to immunoprecipitation of IGF-IR for the detection of tyrosine-phosphorylated IGF-IR and total IGF-IR.

monoculture. Furthermore, leucinostatin A specifically inhibited IGF-I expression even in pseudo-ρ^0 PrSC (Fig. 4*b*).

Discussion

We have recently reported that atpenins including new congeners inhibited the growth of DU-145 cells in coculture with PrSC more strongly than that in monoculture.[23] By further screening, we have found that leucinostatins also showed the same activity as atpenins. These compounds are originally reported as antifungal antibiotics, but they should act as modulators of tumor-stromal cell interactions in our assay system. We therefore examined the possibilities in this study. For *in vivo* evaluation of antitumor activity, we inoculated DU-145 cells along with PrSC subcutaneously, because DU-145 tumors were stimulated by coinoculation of PrSC[14] and the screening system was carried out as coculture of DU-145 cells and PrSC. As a result, leucinostatin A was found to inhibit the tumors of DU-145 cells and PrSC more significantly than atpenin B (Fig. 2*a*). However, the antitumor effect of leucinostatin A *in vivo* was only seen against the coinoculation of PrSC, and it failed to suppress the growth of tumors of only DU-145 cells. *In vitro* leucinostatin A weakly inhibited the growth of DU-145 cells in monoculture, but such direct antitumor activity could not be obtained in the doses used in this study. Although there is a possibility that higher doses of leucinostatin A could suppress the growth of tumors of only DU-145 cells, we could not test it due to its high toxicity in mice. It is previously reported that leucinostatins cannot inhibit Ehrlich ascites tumors, but it weakly suppresses Ehrlich subcutaneous tumors.[30] Thus, it is considered that leucinostatins will show the antitumor effect *in vivo* in a certain condition such as presence of stromal cells. It is reported that p53 mutations in stromal cells augment the sensitivity of tumor cells against some antitumor drugs.[35] However, this is less likely, because PrSC are normal primary cells harboring wild type p53.[36]

DU-145 cells respond to mitogenic action of IGF-I, and the growth of DU-145 cells *in vitro* and *in vivo* is stimulated by IGF-I producing PrSC.[14,33] Many reports showed that IGF-I is one of critical mediators of tumor-stromal cell interactions in prostate.[15] Thus, there is a high possibility that active compounds in our assay system act on the IGF-I signals. In fact, we have recently reported that phthoxazolin A, another active compound in our assay system, inhibited the IGF-I expression in PrSC through suppression of myofibroblast differentiation of PrSC.[22] Although the real contribution of SM α-actin positive myofibroblasts to the tumor promotion is still controversial, the production of IGF-I in stromal cells is critical for growth of some tumors. Therefore, in respect to the mechanism of leucinostatin action we focused on IGF-I axis. As a result, we have found that leucinostatin A specifically inhibited IGF-I expression in PrSC (Fig. 3). Because phthoxazolin A also inhibited expressions of various IGFBPs and SM α-actin in addition to IGF-I, the mechanism