Antitumor activity of pyrvinium pamoate, 6-(dimethylamino)-2-[2-(2,5-dimethyl-1-phenyl-1*H*-pyrrol-3-yl)ethenyl]-1-methyl-quinolinium pamoate salt, showing preferential cytotoxicity during glucose starvation

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An anthelminthic, pyrvinium pamoate (PP), 6-(dimethylamino)-2-[2-(2,5-dimethyl-1-phenyl-1*H*-pyrrol-3-yl)ethenyl]-1-methyl-quinolinium pamoate salt, has been found to be extremely toxic to PANC-1 cells in glucose-free medium, but not to be toxic to the same cells cultured in ordinary medium, Dulbecco's modified Eagle's medium (DMEM). It showed the same preferential toxicity for various cancer cell lines during glucose starvation. When 0.1 μg/ml PP was added to the medium, spheroid growth of human colon cancer cell line WiDr was strongly inhibited to a diameter of 750 μm, and this finding is consistent with the concept of anti-austerity. PP was also found to exert antitumor activity against human pancreatic cancer cell line PANC-1 in nude mice and SCID mice when it was administered subcutaneously or orally. Regarding the mechanism of PP action, inhibition of Akt phosphorylation, which has been found to be essential for the austerity mechanism, was observed *in vitro* and *in vivo*. These findings indicate that PP may be useful for anticancer therapy and that anti-austerity therapy could be a novel strategy for anticancer therapy. (Cancer Sci 2004; 95: 685–690)

Human cancers arise through many steps of carcinogenesis in which multiple genetic alterations, genetic or epigenetic, are often observed.[1, 2] Since the blood supply to cancer tissue provided by tumor angiogenesis is recognized as a critical factor during cancer progression, anti-angiogenesis therapy is now regarded as the most promising approach to cancer therapy.[3, 4] Even conventional cytotoxic agents have recently been found to exert an anti-angiogenic effect when they are effective in treating cancer,[5] and the poor outcome of human hepatoma is closely linked to its ability to form tumor vasculature.[6] Hypoxia dramatically stimulates cell production of angiogenic factors, such as vascular endothelial growth factor (VEGF), through activation of the hypoxia responsive transcription factor, HIF-1,[7, 8] and under hypoxic conditions, glycolysis is markedly stimulated to maintain cellular energy production.[9]

However, angiography often shows human cancers to be hypovascular clinically, and a hypoxic state of the tumor tissue is often associated with greater malignant potential of the cancer.[10–12] One possible explanation for this is that hypoxic episodes are the driving force for cancers to acquire angiogenic ability during progression,[12] and we have hypothesized that the ability to tolerate nutrient starvation, i.e., austerity, is another aspect of the hypoxia response, and therefore a critical characteristic of malignant tumors.[13, 14] This hypothesis is mainly based on the finding that many types of cells become tolerant to glucose starvation under hypoxic conditions, when even more glucose would be expected to be needed for increased glycolysis.[15] There must be an unknown physiological response to insufficient blood supply, when the supply of both oxygen and nutrients, especially glucose, is insufficient. As expected, several cell lines derived from pancreatic cancer, a representative hypovascular tumor, have been found to be tolerant to glucose starvation.[14, 15] These findings suggest that tolerance to nutrient starvation during the progression of hypovascular tumors may be acquired through genetic alterations.

Since the blood supply to normal tissue is delicately regulated by physiological responses, and as a result, chronic ischemia does not occur in normal tissue, we hypothesized that tumor tolerance is a good target for a new cancer therapy strategy, anti-austerity.[13, 14] Based on this hypothesis, we tested several anthelminthics for ability to abolish cancer cells' tolerance to glucose starvation, because some parasites are known to have the ability to produce energy under anaerobic conditions through a specific metabolic pathway during their life cycle, and these mechanisms are sometimes the target of anthelminthics.[16–18]

We newly identified a candidate drug that inhibits cancer cell survival only during nutrient starvation and found that it blocks cellular signaling for cell survival and inhibits tumor formation in nude mice.

Materials and Methods

Chemicals. Pyrvinium pamoate (PP), 6-(dimethylamino)-2-[2-(2,5-dimethyl-1-phenyl-1*H*-pyrrol-3-yl)ethenyl]-1-methyl-quinolinium[16] pamoate salt was obtained from Sigma. Other chemicals were all commercially available reagent-grade products.

Cells and culture. Human pancreatic cancer cell lines, PANC-1, KP-3, and Capan-1, and a human colon cancer cell line, WiDr, were obtained from the Japanese Cancer Research Resources Program and maintained in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo) supplemented with 10% fetal calf serum (FCS, Sigma). To subject the cells to nutrient starvation, various media lacking serum, glucose, and/or amino acids were prepared as described previously.[14] Briefly, nutrient-deprived medium was prepared by removing all carbon sources from DMEM. When glucose was added, D-glucose was added to a final concentration of 1 mg/ml. When amino acids were included, MEM amino acid mixture, containing essential and non-essential amino acids and L-glutamine, was added to the final concentration used in DMEM. FCS was used after exten-

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sive dialysis against saline to remove possible carry-over of amino acids and glucose. Cell survival was assessed either by the dye-exclusion method using Trypan Blue or by the propidium iodide and the Hoechst33342 double-staining method,[19] and cell counts were made either by using Coulter Counter model Z1 or by the WST-8 method (Dojin, Kumamoto, Japan) according to the manufacturer's recommendations.

Spheroid formation and inhibition of spheroid growth. WiDr was used to examine the effect of PP on spheroid formation, because these cells form spheroids more clearly and reproducibly than PANC-1 cells. WiDr cells were added (1000/well) to a Sumilon cell tight spheroid plate (Sumitomo Bakelite, Tokyo, Japan) and cultured in ordinary DMEM containing 10% FCS. After 2 days of culture, the cells in each well formed a spheroid that was approximately 500 μm in diameter. The spheroids were subsequently cultured in either ordinary medium or medium containing 0.1 μg/ml PP. The culture medium was replaced with fresh medium every other day throughout the 28-day experiment, and the diameter of each spheroid was measured by taking a photograph and dividing the circumference measured with image analyzer software, MacSCOPE (Mitani Corp. Tokyo), by 3.14. Measurements were made every 4 days.

Western blot analysis of Akt phosphorylation. PKB/Akt was analyzed by western blotting using antibody against Akt or antibody against the phosphorylated form of Akt at serine 473. Antibodies were obtained from New England Biolab. Cell extracts were prepared by extraction with boiling sample buffer containing 10% sodium dodecyl sulfate (SDS), 1 *M* 2-mercaptoethanol, 50 m*M* Tris-HCl buffer (pH7.5) as described previously.[14] Approximately 20 μg of the extract protein was loaded onto each lane, and the protein was transferred to a nylon membrane and blocked with 2% skim milk. The membrane was washed three times with TBS (0.2% Tween 20, 150 m*M* NaCl, and 50 m*M* Tris-HCl(pH7.5), incubated for 24 h at 4°C with 1000-fold diluted antibody, and then incubated again with second antibody against rabbit immunoglobulin conjugated with horseradish peroxidase (Amersham). Bands were visualized with a Luminescence kit (Amersham). When tumor tissues were analyzed, proteins were extracted from subcutaneous tumors for immunoblotting analysis. The tumors removed from the mice were dissected and crushed with a metal crusher, frozen in dry ice, and then cut into small pieces with scissors, vortexed for 1 h in 8 *M* urea containing 1% SDS and 2.5% dithiothreitol (DTT, Sigma), and vortexed again for 20 min after addition of 10% NP-40. The extracts were obtained by centrifugation at 15,000 rpm, and the protein concentration in the supernatant was determined with a BCA protein assay kit (Pierce).

***In vivo* antitumor activity.** PANC-1 cells were cultured *in vitro* with DMEM supplemented with 10% FCS and then subcutaneously injected into nude mice (BALB/cAnCrj-nucrj, Charles River Japan, Yokohama, Japan) or Severe Combined Immunodeficiency (SCID) mice (C.B-17/IcrCrj-scid, Charles River Japan) at a dose of 5×10^6 cells/mouse. Small tumors appeared after about 2 weeks, and the mice were randomly divided into two groups in a manner that minimized the difference in tumor size between the groups. When SCID mice were used, groups were established 3 weeks after transplantation. PP was suspended to a concentration of 200 or 400 μg/ml in 2% DMSO in saline, and the mice were force-fed intragastrically with 0.5 ml of the suspension or vehicle, 6 days a week, until the end of the experiment. In the preliminary experiment by subcutaneous administration, PP dissolved to a concentration of 50 μg/ml in 2% DMSO in saline or vehicle was subcutaneously injected into the contralateral side of the back, 6 days a week, until the end of the experiment. The mice were weighed weekly, and the tumors on the back of each mouse were measured weekly with vernier calipers. Tumor size was calculated by using the following formula: $V=4/3\times\pi\times(L/2\times W/2\times W/2)$, where L is length and W is width. At the end of the experiment, the tumors were resected and processed for histological examination. Formalin-fixed paraffin-embedded sections were stained with hematoxylin and eosin.

Immunohistochemistry. Purified rat anti-mouse CD31 (PECAM-1) monoclonal antibody (BD Biosciences) was used to detect tumor vasculature immunohistochemically. Briefly, the subcutaneous tumors were removed after sacrifice of the mice at the end of the experiment, embedded in O.C.T compound (Tissue-Tek; Sakura Finetechnical Co., Tokyo), and frozen, and 4-μm thick serial sections were prepared. The tissue sections were fixed with 100% acetone and Carnoy's fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 15 min and then immersed in methanol containing 0.3% hydrogen peroxide to block endogenous peroxide activity. After incubation with a blocking buffer (2% BSA in PBS buffer), the sections were exposed to the first antibody (100-fold dilution) for 1 h at room temperature. Biotinylated polyclonal anti-rat IgG (BD Biosciences) was used as the secondary antibody. Peroxide staining was performed for 2–5 min with a solution of 3,3′-diaminobenzidine tetrahydrochloride in 50 m*M* Tris-HCl (pH 7.5) containing 0.001% hydrogen peroxide, and the sections were counterstained with 0.1% hematoxylin. Intratumoral microvessel density was analyzed with a KS300 imaging system (Carl Zeiss Vision, GmbH). Photographs of the entire area of 10 slides from tumors in the treated group and control group were taken after staining, and all of the vessels were counted on each slide with the KS300 imaging software. Microvessel density was calculated as the total number of vessel on each slide/the gross area of the slide.

Results

***In vitro* cytotoxicity of PP.** PANC-1 cells which were originally derived from a human pancreatic cancer, a representative hypovascular tumor, showed strong tolerance to glucose starvation *in vitro*, as found previously.[15] In the presence of 0.1 μg/ml of PP, the cells grew almost normally in ordinary DMEM medium, though cell growth slowed at 1 μg/ml (Fig. 1A). Strong cell killing was observed in nutrient-deprived medium, and even 0.1 μg/ml was sufficient to kill all of the cells within 24 h. To determine the dose-response relationship of PP cytotoxicity, various concentrations of PP were tested for cytotoxicity during a 24-h period. The results showed that 0.1 μg/ml was adequate to kill most of the cells (Fig. 1B). These results were obtained by both the Trypan Blue dye exclusion method and cell counting with a Coulter Counter, and by the propidium iodide and Hoechst33342 double-staining method in some cases. All the data were highly consistent. The PANC-1 cells were examined for morphological changes during culture in nutrient-deprived medium in the presence of 0.1 μg/ml PP by staining with propidium iodide and Hoechst33342. Most of the cells were swollen at 3 h, and by 6 h had become positive for propidium iodide staining and lacked convoluted nuclei or fragmented chromatin, indicating necrotic death. However, little cell death was observed at 24 h in DMEM, irrespective of PP treatment, or in nutrient-deficient medium without PP (Fig. 1C).

PP is preferentially toxic when glucose is depleted. The effect of PP on PANC-1 cell survival under various nutrient-starved conditions was investigated by removing glucose, amino acids, and/or serum. As shown in Fig. 2, 1 μg/ml of PP was toxic when glucose was depleted, irrespective of the presence of serum and amino acids. However, it had little effect on cell survival when glucose was present.

PP was toxic to various cell lines during glucose starvation. To determine whether PP is toxic to only PANC-1 cells during glucose starvation, its effect was tested on other pancreatic cancer

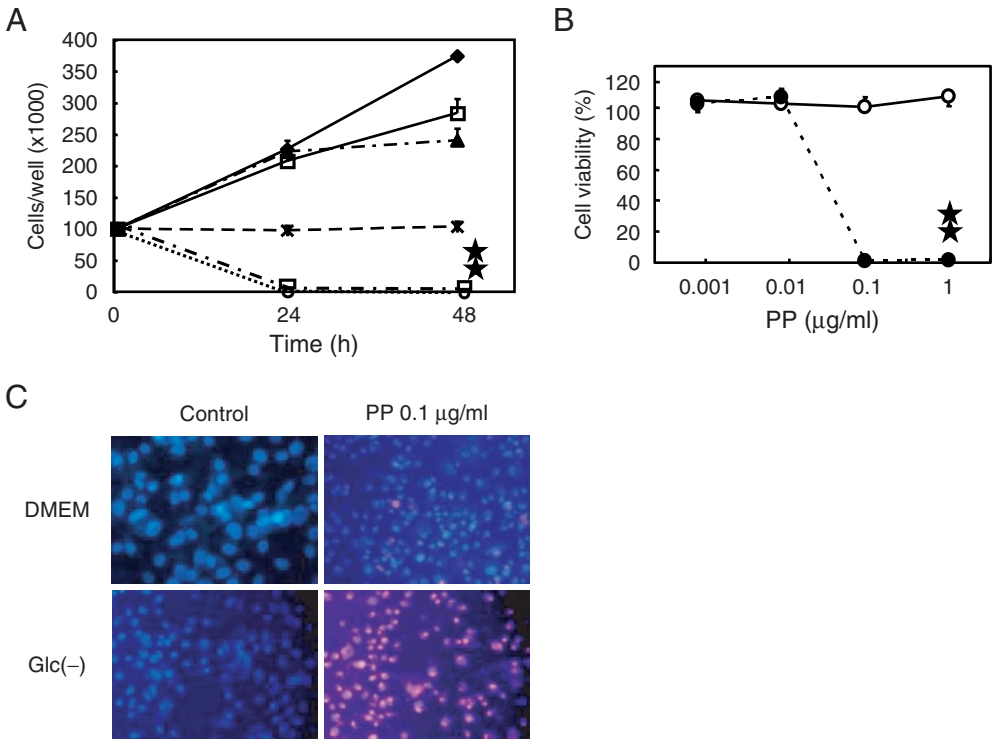

Fig. 1. Cytotoxicity of PP. PANC-1 cells were cultured either in the nutrient-deprived medium or DMEM with or without PP (pp) at atmospheric oxygen tension. Surviving cells were identified by the Trypan Blue dye exclusion method and cells were counted with a Coulter Counter. A, Time course of the effect of PP on cell growth and survival. ◆—◆, DMEM without PP; □—□, DMEM with 0.1 μg/ml PP; ▲– –▲, DMEM with 1 μg/ml PP; ×– –×, nutrient-deprived medium without PP; □– –□, nutrient-deprived medium with 0.1 μg/ml PP; ○– –○, nutrient-deprived medium with 1 μg/ml PP. B, Effect of various concentrations of PP on PANC-1 cell survival. The number of cells cultured in DMEM without PP was set equal to 100%. Cell number was counted 24 h after treatment. ○—○, cells cultured in DMEM; ●—●, cells cultured in nutrient-deprived medium. C, Propidium iodide and Hoechst33342 double-staining pattern 24 h after treatment with 0.1 μg/ml PP. Photographs were taken through fluorescence microscope 24 h after the start of the treatment as described elsewhere.[19] Glc(–), cells cultured in DMEM without glucose. ★ ★, statistically significant difference from the control at $P<0.01$.

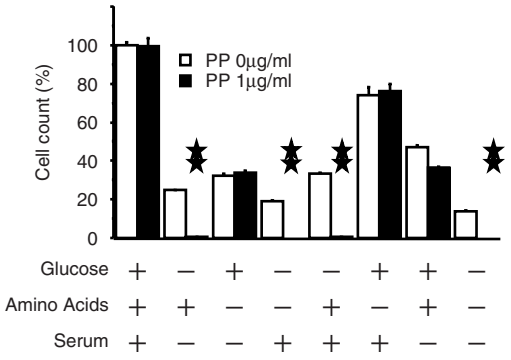

Fig. 2. Cytotoxicity of PP in various media lacking different nutrient components. PANC-1 cells were cultured for 24 h at atmospheric oxygen tension, and the surviving cells were counted by the WST-8 method. Glucose was added at 1 mg/ml. The amino acids were a mixture of all amino acids at the same concentrations as in DMEM. Extensively dialyzed serum was added at a 10% concentration. Cell number detected by WST-8 method in DMEM without PP was defined as 100%. PP was added at 1 μg/ml. ★ ★, statistically significant difference from the corresponding control without PP at $P<0.01$.

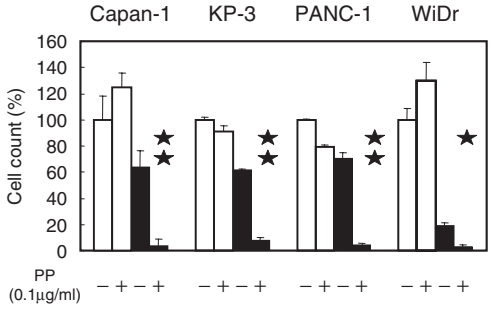

Fig. 3. Cytotoxicity of PP for various cell lines. Human pancreatic cancer cell lines Capan-1, KP-3, and PANC-1 and human colon cancer cell line WiDr were cultured with either DMEM (open bar) or glucose-free medium (closed bar), with (+) or without 0.1 μg/ml PP (–). Surviving cell were counted by the WST-8 method. In some cases, cell death was confirmed morphologically after staining with propidium iodide and Hoechst33342. The results are means and SD of three wells for each point. ★, statistically significant difference from the corresponding control without PP at $P<0.05$. ★ ★, statistically significant difference from the corresponding control without PP at $P<0.01$.

cell lines, KP-3 and Capan-1, and a human colon cancer cell line, WiDr. Although cell survival after 24-h glucose starvation varied among the cell lines, as noted previously,[15] the results in Fig. 3 clearly show that PP was toxic to all of the cell lines tested only in the absence of glucose, and not in its presence.

PP strongly inhibits spheroid growth. Oxygen and nutrient supply in three-dimensional cancer tissue is determined by their rate of supply to the tissue, their rate of consumption by the tissue components, and their rate of diffusion. Because most cancer cell tissue cultures are monolayers, it is difficult to simulate the effect of agents that are selectively toxic during nutrient deficiency under ordinary culture conditions. Spheroids are an *in vitro* model of the structural organization of tumor tissue, and

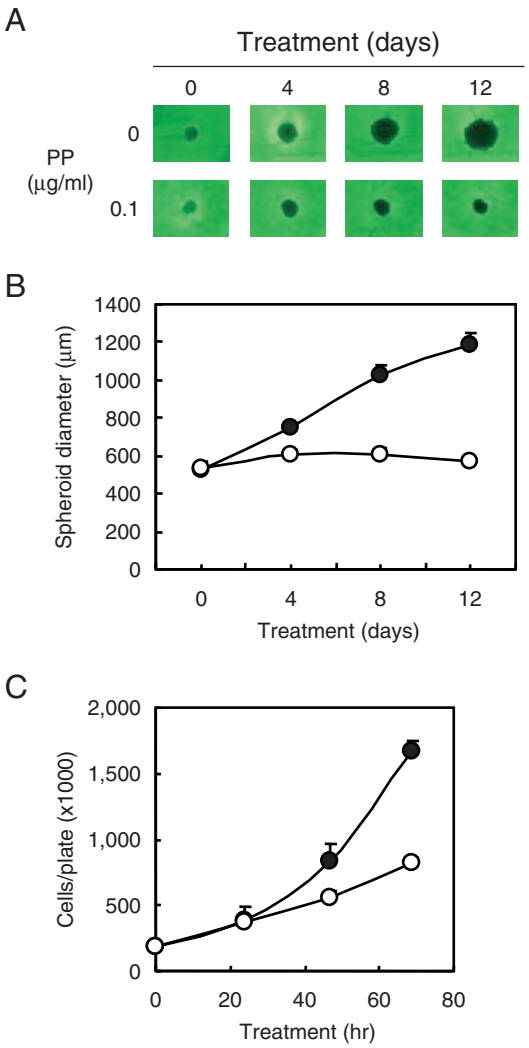

Fig. 4. Effect of PP on spheroid growth of human colon cancer cell line WiDr. A, Representative photograph of spheroids that formed in the presence and absence of 0.1 μg/ml PP. Photographs were taken through a phase-contrast microscope. B, Growth of spheroids during treatment. Diameters were calculated by measuring the circumference of the spheroids on photographs. The results are expressed as means and SD calculated from the data for at least 10 spheroids from each treatment. ●—●, spheroids cultured in DMEM without PP; ○—○, spheroids cultured in DMEM with 0.1 μg/ml PP. C, Cell growth curves of WiDr cell culture on ordinary tissue culture dishes in DMEM. The results are means and SD of three wells for each point. ●—●, cells cultured in DMEM without PP; ○—○, cells cultured in DMEM with 0.1 μg/ml PP.

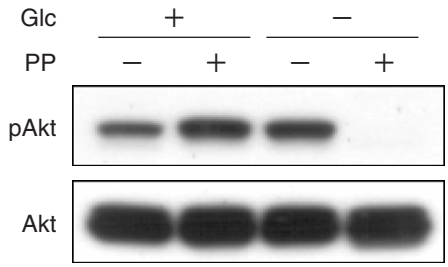

Fig. 5. Western blot analysis of the effect of PP on Akt activation. PANC-1 cells were cultured either in ordinary DMEM (Glc +) or DMEM without glucose (Glc−) in the absence (−) or presence (+) of 1 μg/ml PP. Cells were harvested 2 h after the start of the treatment. Phosphorylated Akt (pAkt) and total Akt (Akt) proteins were detected by western blot analysis with specific antibodies.

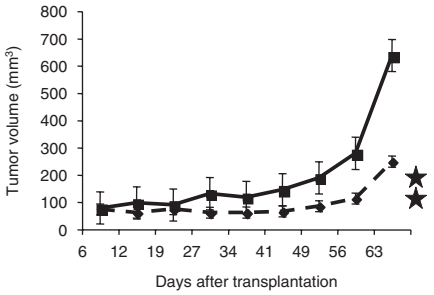

Fig. 6. Antitumor activity of PP as measured by effect on PANC-1 cell tumor formation in nude mice. Six days after transplantation of the tumor cells, animals were divided into two groups, and treatment 6 days/week was started 2 weeks after transplantation. PP (◆– –◆) or vehicle (■—■) was force-fed intragastrically. Tumors were measured every week, and tumor volume was calculated as described in "Materials and Methods." Each point is a mean and SD of data from 10 mice. ★ ★, statistically significant difference from the control without PP at $P<0.01$.

oxygen and nutrients availability is known to be very limited at the center of a spheroid.[20, 21] Because human colon cancer cell line WiDr is often used for spheroid experiments, we used it to produce spheroids in this study. When 1000 cells/well were seeded onto a 96-well Sumilon cell tight spheroid plate, spheroids about 500 μm in diameter formed within 2 days. The size of the spheroids increased to about 2000 μm in diameter within 28 days of culture during which time the medium was replaced with fresh medium every other day. However, when 0.1 μg/ml of PP was included in the same medium, the spheroids stopped increasing in size after the initial 4 days, growing to about 750 μm by the end of the experiment without obvious cell death (Fig. 4, A and B). The effect of PP 0.1 μg/ml on the growth of WiDr cells in monolayer culture was also investigated, and it was found to be slightly inhibitory, increasing the doubling time about twofold (Fig. 4C).

PP blocks Akt phosphorylation by glucose starvation. In our previous work we found that PKB/Akt is activated within 5–10 min of the depletion of glucose in the medium and that PKB/Akt is essential for the survival of cells during glucose starvation both under normoxic and hypoxic conditions.[13, 14] The effect of PP on PKB/Akt activation was investigated and, as shown in Fig. 5, PKB/Akt phosphorylation in DMEM was slightly stimulated by PP in the presence of glucose, whereas phosphorylation of PKB/Akt at serine 473 was completely blocked by PP under conditions of glucose starvation that otherwise would have stimulated Akt phosphorylation.

PP strongly inhibits tumor formation by PANC-1 cells in nude mice. PP is used clinically as an anthelminthic. Since it is barely soluble in water and has been reported to be hardly absorbed by the mammalian intestine,[22] and would therefore seem difficult to administer orally to treat tumors in nude mice, it was administered subcutaneously first. One side of the back of each nude mouse was subcutaneously injected with 5×10^6 PANC-1 cells in a preliminary study, and the results demonstrated antitumor activity of PP against PANC-1 cells (data not shown). Although PP has been claimed not to be absorbed by the mammalian intestine, preliminary tests indicated that it is absorbed, because the urine of a human turned red after oral consumption of 100 mg of a commercial anthelminthic preparation (our unpublished observation). We examined the effect of orally administered PP on tumor growth in nude mice bearing PANC-1 tumors. The results (Fig. 6) clearly demonstrate that PP exerted clear antitumor activity on the PANC-1 cells in nude mice. The dose of PP

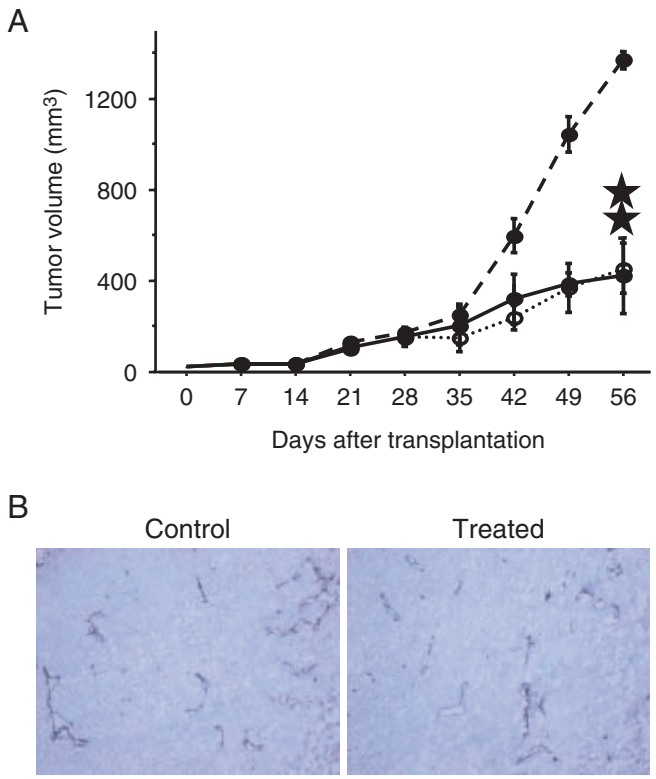

Fig. 7. Antitumor activity of orally administered PP against PANC-1 cell tumors in SCID mice. A, PP was administered orally at a dose of 100 μg (○—○) or 200 μg/mouse 6 days/week (●—●) until the end of the experiment. Oral administration of PP was achieved by force-feeding a 200 μg/ml or 400 μg/ml PP suspension in 2% DMSO in saline through a force-feeding syringe without anesthesia. Each group consists of 9 mice. Control mice received the same volume of vehicle (●– –●). Treatment was started 3 weeks after transplantation. ★ ★, statistically significant difference from the control without PP at $P<0.01$. B, Representative tumor vasculature detected by CD31 immunohistochemistry. Histological sections from animals treated with vehicle (Control) and PP (Treated) were immunohistochemically stained with anti CD31 antibody, and a photograph was taken through a microscope at ×100 magnification.

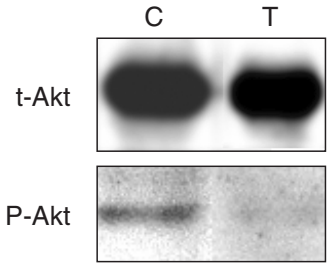

Fig. 8. Western blot analysis of PKB/Akt activation in tumors from mice given or not given PP orally. PANC-1 cells were transplanted into nude mice, and they were fed basal diet for 6 weeks. Two hours before sacrifice, the mice were given either 100 μg of PP (T) or vehicle (C), and the tumor tissue was immediately frozen with liquid nitrogen and subjected to western blot analysis of total Akt (t-Akt) or the phosphorylated form of Akt (p-Akt).

administered was 100 μg/mouse/day.

Oral administration of PP inhibits tumor formation by PANC-1 cells in SCID mice. To confirm the antitumor activity and to assess the effect of the dose of PP, the *in vivo* experiment was repeated in SCID mice and PP was administered at the dose of either 100 μg/mouse/day or 200 μg/mouse/day. The results shown in Fig. 7A clearly demonstrate that the antitumor activity is reproducible. The 100 μg/day dose clearly suppressed tumor growth and the 200 μg/day was equally effective, indicating saturation of the effect. The body weights of animals given vehicle and PP, both 100 and 200 μg/day, did not show any significant differences, indicating low toxicity of PP (data not shown).

Histological examination of the tumors treated with PP revealed that the residual tumor was not necrotic. The microvessel densities of the tumors from control animals and treated animals were compared after staining sections with antibody against CD31 (Fig. 7B), and the values were 184.2±33.5 and 182.5±31.6 (mean±SE, number of vessels/mm^2), respectively.

Orally administered PP inhibits Akt phosphorylation *in vivo*. Because PP had been reported not to be absorbed by the mammalian intestine, we attempted to determine whether PP actually reached the tumor tissue by investigating the effect of PP on Akt phosphorylation *in vivo*. As shown in Fig. 8, the phosphorylated form of Akt in tumor tissue was clearly decreased by oral administration of PP given 2 h before sacrifice, indicating that PP had been absorbed and had reached the tumor tissue.

Discussion

The results of this study clearly demonstrate antitumor activity of PP in nude mice and SCID mice. Tumor formation was markedly suppressed by PP, but not completely, even at the higher dose. The remaining tumor cells were as viable as untreated tumor cells and were not necrotic, probably because of the effect of PP being toxic to tumor cells only during glucose starvation.

PP was found to inhibit the growth of WiDr cell spheroids rather than their formation. The inhibition of spheroid growth may be attributable to the inhibition of cell growth, and PP actually had a moderately inhibitory effect on WiDr cell growth in culture dishes. Because the inhibition of cell growth was partial and PP completely inhibited spheroid growth at a certain size, the effect of PP on spheroid growth cannot be explained simply by inhibition of cell growth. Based on the above two findings, together with the preferential toxicity of PP during glucose starvation, we propose that PP exerts its antitumor effect through preferential toxicity against tumor tissue in the glucose-starved condition, namely, anti-austerity.

The molecular and biochemical mechanisms of the antitumor activity of PP cannot be fully understood based on the results of this study, but for the reasons given above it is likely that PP acts by inhibiting tumor cell survival in the presence of an insufficient blood supply. PP exerted selective toxicity against various cancer cells only during glucose starvation. PP toxicity was observed when the glucose concentration in the culture medium was less that 0.2 mg/ml. The glucose concentration in the interstitial fluid of xenograft of human pancreatic cancer cell lines is less than 0.2 mg/ml (our unpublished data). It is interesting that the microvessel density in the residual tumors of nude mice after long-term PP administration was almost the same as in untreated tumors and no prominent necrosis was observed in treated tumors. Interestingly, the bromodeoxyuridine labeling indexes of PP-treated tumors were sometimes the same as or higher than those of untreated tumors, indicating a dynamic balance between cell growth and death in the tumors with smaller tumor volume treated with PP (our unpublished data). We recently discovered a novel compound, kigamicin D,[23, 24] that exhibits selective toxicity during nutrient starvation and an antitumor effect that are similar to those of PP. Kigamicin D treatment causes a similar dynamic balance between cell growth and death in smaller tumors *in vivo*, and a similar effect on spheroid growth was also observed.[25]

One clear biochemical effect of PP identified in this study

was inhibition of the Akt phosphorylation stimulated by glucose starvation. Inhibition of Akt activation is likely to be one of biochemical mechanisms of the antitumor effect of PP, because Akt has been found to be involved in tumor cell tolerance to glucose starvation.[13, 14, 26] However, it is important to note that a representative PI3 kinase inhibitor, LY294002, displayed preferential toxicity only under different conditions, despite inhibiting the Akt phosphorylation caused by glucose starvation.[14] Moreover, PP inhibited the Akt phosphorylation induced by glucose starvation even at 0.1 μg/ml, but it did not inhibit Akt phosphorylation by insulin or IGF-1 even at 1 μg/ml (to be published). These results raised doubts about the simple interpretation that Akt inhibition is the mechanism of its antitumor effect.

The biochemical mechanisms underlying the anthelminthic action of PP are not fully understood. Inhibition of glycogen and glucose utilization by worms has been reported.[27, 28] Parasites often produce energy through a fumarate reductase reaction that catalyzes the reduction of fumaric acid to succinic acid under anaerobic conditions.[18, 29, 30] We recently found that PP inhibits the fumarate reductase activity of worms *in vitro* (Miyadera H. *et al*. to be published elsewhere). There is a report indicating that fumarate reductase activity may be important in the response of mammalian cells to hypoxia, although the enzyme itself has not yet been identified.[31] Cancer cell utilization of enzyme activity that is phylogenically conserved among anaerobic organisms is a very interesting possibility, but it remains to be determined whether the inhibitory activity of PP is related to the biochemical mechanism of its antitumor activity.

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