Kigamicin D, a novel anticancer agent based on a new anti-austerity strategy targeting cancer cells' tolerance to nutrient starvation

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Both tolerance to nutrient starvation and angiogenesis are essential for cancer progression because of the insufficient supply of nutrients to tumor tissue. Since chronic nutrient starvation seldom occurs in normal tissue, cancer's tolerance to nutrient starvation should provide a novel target for cancer therapy. In this study, we propose an anti-austerity strategy to exploit the ability of agents to eliminate cancer cells' tolerance to nutrient starvation. We established a simple screening method for agents that inhibit cancer cell viability preferentially during nutrient starvation, using PANC-1 cell line cultured in nutrient-rich and nutrient-deprived media. After screening over 2000 culture media of *actinomycetes*, we identified a new compound, kigamicin D ($C_{48}H_{59}NO_{19}$), which shows preferential cytotoxicity to cancer cells under nutrient-deprived conditions, but hardly any cytotoxicity under nutrient-rich conditions. Both subcutaneous and oral administration of kigamicin D strongly suppressed the tumor growth of several tested pancreatic cancer cell lines in nude mice. Moreover, kigamicin D was observed to block the activation of Akt induced by nutrient starvation. Therefore, our results suggest that kigamicin D be a candidate for implementing our novel concept, anti-austerity, which may serve as a new strategy for cancer therapy. (Cancer Sci 2004; 95: 547–552)

Cancers always have deformed, deficient vascular and lymphatic systems due to excessive and unregulated tumor cell growth, as well as improper and insufficient angiogenesis. As a result, perfusion within cancers is inadequate, and the cancers contain regions that are transiently and chronically exposed to nutrient starvation.[1)] These microenvironmental inadequacies are present from the earliest stage in the development of cancers, and are fully established while the neoplasm is still microscopic in size.[2)] Since nutrient starvation occurs in tumor tissue that is >100–200 μm away from a functional blood supply,[3)] tumor survival depends, in part, on the ability to recruit new blood microvessels via angiogenic factors. This affords tumor cells the ability to survive and propagate in a hostile environment,[4)] and thus increased angiogenesis by a malignancy is related to a poor prognosis.[5,6)] Because of this, antiangiogenic therapy was postulated to be the most promising and specific approach to cancer therapy. Already, extensive studies have been conducted in an attempt to prevent tumor angiogenesis.

However, Yu *et al*. recently suggested that tumors may be able to elude the effect of angiogenesis inhibitors, since antiangiogenic therapy targets genetically stable endothelial cells in the tumor vasculature, and genetic alterations that decrease the vascular dependence of tumor cells can influence the response of tumors to this therapy.[7)]

The well-known observation that hypovascularity is an outstanding characteristic of pancreatic cancers in diagnostic images suggests that pancreatic cancer tissue has a poor blood supply.[8)] However, since pancreatic cancers are known to be among the most aggressive malignancies despite their poor blood supply, we proposed that cancer cells' tolerance of insufficient nutrient might be an important determinant of malignancy.[9)] Although the mechanism of tolerance has not yet been fully elucidated, the PI-3K-Akt pathway and AMPK have been found to be involved.[10)]

Because the blood supply of normal tissue is regulated in a sophisticated manner, angiogenesis is seldom activated in normal healthy adult tissues, except in the female reproductive system.[11)] The same is true for nutrient starvation. We therefore speculated that if some compound could abolish cancer cells' tolerance of nutrient starvation, it might be capable of being used to sensitize cancer cells to this hostile environment, resulting in repression of cancer by a new strategy that we termed "anti-austerity."[12)]

In this study, through screening over 2000 culture media of *actinomycetes*, we identified a new compound, kigamicin D, a polycyclic xanthone having sugar moieties and a molecular formula of $C_{48}H_{59}NO_{19}$, as a candidate for this strategy. The possible mode of action of kigamicin D was also investigated.

Materials and Methods

Cells and culture. Human cancer cell lines, PANC-1, Capan-1 and MIA PaCa-2 (pancreatic cancer), SW-480 (colon cancer) and A431 (skin cancer) obtained from American Type Culture Collection were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo) supplemented with 10% fetal calf serum (Sigma, MO), 2% L-glutamine, 1% penicillin, and 1% streptomycin stock solutions. Twenty percent fetal calf serum was used for Capan-1 cell. The medium was routinely changed every 3 days, and cells were passaged by trypsinization before confluence. Nutrient starvation was achieved by culturing with nutrient-deprived medium as previously described.[9)] Briefly, the nutrient-deprived medium was prepared as follows: $CaCl_2(2H_2O)$, 265 mg/liter; $Fe(NO_3)(9H_2O)$, 0.1 mg/liter; KCl, 400 mg/liter; $MgSO_4(7H_2O)$, 200 mg/liter; NaCl, 6400 mg/liter; $NaHCO_3$, 700 mg/liter; NaH_2PO_4, 125 mg/liter; phenol red, 15 mg/liter; 25 m*M* HEPES buffer (pH 7.4); and MEM vitamin solution (Life Technologies, Inc., Rockville, MD), then the final pH was adjusted to 7.4 with 10% $NaHCO_3$. When the medium was supplemented with glucose, D-glucose was added at a concentration of 1 mg/ml. A 200 m*M* L-glutamine solution, MEM amino acids solution, and MEM nonessential amino acids solution (Life Technologies, Inc.) were used as stock solution, and were added at 1% concentration when amino acids were included. The fetal calf serum (Sigma) was dialyzed three times against large excesses of

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; IHC, immunohistochemistry; PKB, protein kinase B; PI-3K, phosphatidylinositol 3-OH-kinase; NDM, nutrient-deprived medium.

0.9% NaCl before use.

Conventional anticancer drugs. Vincristine, 5-fluorouracil, doxorubicin, cisplatin, campothecin, and taxol were purchased from Sigma.

Assay and purification of the anticancer agent. The *actinomycetes* culture media were obtained by centrifugation at 3000*g*, and the supernatants were heated to 95°C for 15 min. PANC-1 cancer cells were seeded in 96-well plates (1×10^4/well) and incubated in fresh DMEM medium at 37°C under a 5% CO_2/95% air atmosphere for 24 h. The cells were then washed with PBS buffer, and the medium was changed to either DMEM or nutrient-deprived medium followed by immediate addition of serial dilutions of the test samples. After 24 h incubation, the cells were washed with PBS again, then 100 µl of DMEM medium with 10% WST-8 solution was added to the well, and the plate was incubated for a further 2 h. Then the absorbance at 460 nm was measured. Since the absorbance is proportional to the number of viable cells in the medium, the viable cell number can be determined using a previously prepared calibration curve (Dojindo Co., Kumamoto). The samples that were highly and selectively cytotoxic to cancer cells in nutrient-deprived medium were subjected to further purification. The process of purification and the determination of the molecular structure were described in detail in other papers.[13, 14]

Flow cytometric analysis. For flow cytometric analysis, PANC-1 cells were exposed to 0.1 µg/ml kigamicin D for 6 h, then both floating and trypsinized adherent cells were collected, washed with PBS, and incubated with 2.5% annexin V in binding buffer for 10 min, then with 2.5% propidium iodide in binding buffer (Bender Medsystems, Austria). Stained cells were analyzed using a fluorescence-activated cell sorter (Caliber, Becton Dickinson, Tokyo).

Tumorigenesis in nude mice. Because kigamicin D is poorly soluble in water, it was firstly dissolved in DMSO at 10 mg/ml, and kept frozen until usage. Just before administration, the stock solution of kigamicin D was diluted in saline to give the final concentration of 15 µg/ml (for subcutaneous administration) or 30 µg/ml (for oral administration). Female 5-week-old SPF/VAF BALB/cAn Ncrj-*nu*/*nu* mice were obtained from Charles River Japan, and 5×10^6 cancer cells in 0.3 ml of DMEM were subcutaneously injected into the right lateral abdominal wall. Two weeks later, 24 mice bearing tumors around 5 mm in diameter were randomly divided into a treatment group and a vehicle group in each experiment. In the subcutaneous administration experiment, 0.2 ml of either kigamicin D solution or vehicle was injected subcutaneously into the mice, 6 days/week; in the oral administration experiment, 0.5 ml of either kigamicin D solution or vehicle was force-fed intragastrically, 6 days/week, until the end of the experiment. Tumor size and body weight were measured weekly, and tumor volume was calculated by using the following formula: tumor volume=$4/3\times3.14\times(L/2\times W/2\times W/2)$, where L is the length of the tumor and W is its width.

Immunohistochemistry. The effect of kigamicin D on angiogenesis in the xenografts was investigated immunohistochemically. Purified rat anti-mouse CD31 (PECAM-1) monoclonal antibody (BD Biosciences) was used to detect tumor vasculature immunohistochemically. Briefly, the subcutaneous tumors were removed at the end of *in vivo* experiments, embedded in O.C.T compound (Tissue-Tek; Sakura Finetechnical Co., Tokyo); and frozen, then 4-µm-thick serial sections were microdissected with a Tissue-Tek Cryo (Tissue-Tek; Sakura Finetechnical Co.). The tissue sections were fixed with 100% acetone and Carnoy's fixative (60% absolute 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 (1% dilution) for 1 h at room temperature. Biotinylated polyclonal anti-rat Ig (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 (MVD) was analyzed with a KS300 imaging system (Carl Zeiss Vision GmbH). Briefly, photographs of the entire area of 12 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 vessels on each slide/the gross area of the slide.

Protein extraction. Proteins were extracted from the subcutaneous tumors for immunoblotting analysis. The tumors removed from 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).

Western blot analysis. The proteins were separated by gel electrophoresis on a polyacrylamide gel containing SDS, and then transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 5% (w/v) skim milk, washed with PBS containing 0.3% Tween 20 (Sigma), then incubated overnight at room temperature with Akt antibody and the phospho-specific (Ser-473) Akt antibody (New England BioLabs, MA) diluted with PBS. After washing, the membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, CA) as the second antibody. The bands were detected with an ECL system (Amersham Biosciences UK Ltd., UK).

Statistical analysis. All results are expressed as means±SD. Statistical comparisons were made using Student's *t* test after analysis of variance. The results were considered to be significantly different if P value <0.05.

Results

Preferential cytotoxicity of kigamicin D to cancer cells under nutrient-deprived condition. After screening over 2000 samples, we previously identified a new compound, kigamicin D, $C_{48}H_{59}NO_{19}$ (MW 953) (Fig. 1), as a pale yellow powder, soluble in $CHCl_3$, EtOAc, MeOH, and DMSO, but hardly soluble in water.[13, 14]. As shown in Fig. 2A, kigamicin D had no toxic effect on PANC-1 cancer cells in DMEM, but it completely killed these cells in a nutrient-deprived medium (IC_{50} 85 ng/ml). Fig. 2B shows that the cytotoxicity of kigamicin D in nutrient-deprived medium is time-dependent, and 0.1 µg/ml kigamicin D completely killed PANC-1 cells within 6 h. Kigamicin D also exhibited strong cytotoxicity against several other cancer cell

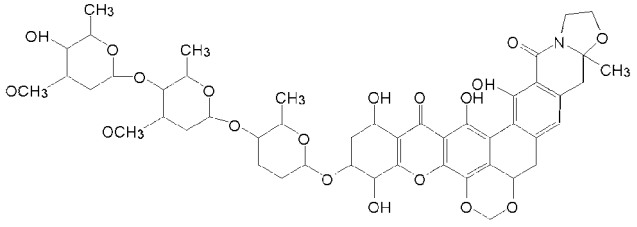

Fig. 1. Structure formula of kigamicin D.

lines tested in nutrient-deprived medium, but not in DMEM (Fig. 2C). To determine which nutrient component's absence was most important for kigamicin D's selective cytotoxicity, we cultured the PANC-1 cell line in medium free of either glucose or amino acids or serum, or two of them, then tested the cell viability with or without 0.05 μg/ml kigamicin D. The results, shown in Fig. 2D, clearly demonstrate that glucose is the key component that determines the sensitivity of cancer cells to kigamicin D cytotoxicity. Interestingly, we found that when serum was supplemented with glucose, the serum seemed to counteract the protective effect of glucose. The mechanism of this action needs further investigation.

Kigamicin D induces necrosis during nutrient starvation. The mode of cell death caused by kigamicin D was examined by the propidium iodide and Hoechst 33342 double-staining method as previously described[15] and FACS analysis using annexin V and propidium iodide staining. When the cells were stained with propidium iodide and Hoechst 33342 after having been treated with kigamicin D under nutrient starvation, most of the cells were stained with propidium iodide without nuclear fragmentation or chromatin condensation, similar to cells treated with DMSO (Fig. 3A), which was used as the control of necrosis,[16] indicating the occurrence of necrotic cell death.[15] These cells showed positive staining with propidium iodide without annexin V staining on FACS analysis, at least in their early stage of death (Fig. 3B). These results clearly showed that kigamicin D induced necrosis of cancer cells under conditions of nutrient starvation.

***In vivo* antitumor effect of kigamicin D.** Because kigamicin D kills cancer cells *in vitro*, we assessed its antitumor effect *in vivo* by using human xenografts as described in the method section. PANC-1 cells (5×10^6) were subcutaneously implanted into nude mice. Fourteen days later the mice with a tumor of around 5 mm in diameter were randomly divided into the treatment group and the control group, and administration was started as described in "Materials and Methods." As shown in Fig. 4A, subcutaneous administration of kigamicin D suppressed the tumor growth in nude mice to about 41%. In addition, oral administration of 15 μg/day/mouse of kigamicin D also suppressed the tumor to about 20% (Fig. 4B), though no significant body weight loss was observed in the treated mice (treated 24.6±1.63 g; control 26.6±2.1 g at the sacrifice time).

To confirm kigamicin D's antitumor effect, we tested 2 other cancer cell lines, MIA Paca-2 (Fig. 5A) and Capan-1 (Fig. 5B) in nude mice. The result clearly showed that oral administration of kigamicin D also significantly inhibited these cell lines in animals.

Since growth of transplanted tumors largely depends on the

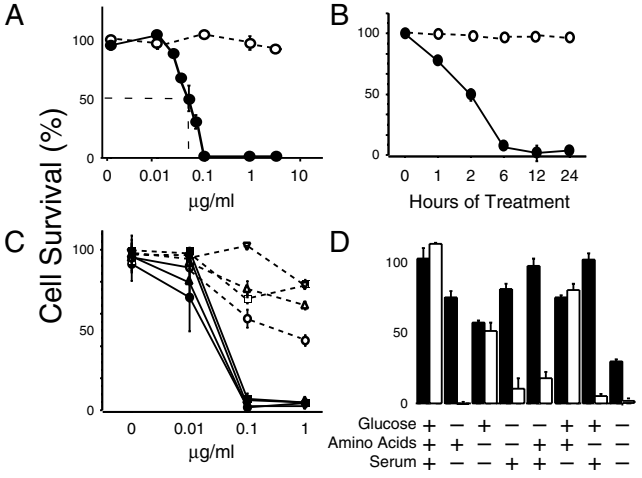

Fig. 2. Kigamicin D preferentially kills cancer cells under nutrient-deprived conditions. Cancer cells were seeded at a density of 1.0×10^4 cells/well in 96-well plates and incubated for 24 h in completely fresh medium containing 0–5 μg/ml kigamicin D. The viable cell number was determined using the absorbance value of a previously prepared calibration curve with the WST-8 cell counting kit. The viability is shown as the percentage of the viable cell number in wells treated with kigamicin D versus untreated wells. A. Dose-dependent effect of kigamicin D on PANC-1 cell viability. The normal medium, DMEM (○); nutrient-deprived medium (●). B. Time-dependent effect of kigamicin D (0.1 μg/ml) on PANC-1 cell viability. C. Kigamicin D's preferential cytotoxicity against other cancer cell lines. ○ HepG2 in DMEM, ● HepG2 in nutrient-deprived medium, △ SW480 in DMEM, ▲ SW480 in nutrient-deprived medium, □ HLE in DMEM, ■ HLE in nutrient-deprived medium, ▽ Capan-1 in DMEM, ▼ Capan-1 in nutrient-deprived medium. D. Each nutrient component, glucose, amino acid or serum was added to the nutrient-deprived medium, then 0.05 μg/ml kigamicin D was added to the cells. Cell viability was calculated 24 h later. ■ without kigamicin D, □ with 0.05 μg/ml kigamicin D. All experiments were performed in triplicate, and the results are shown as means±SD.

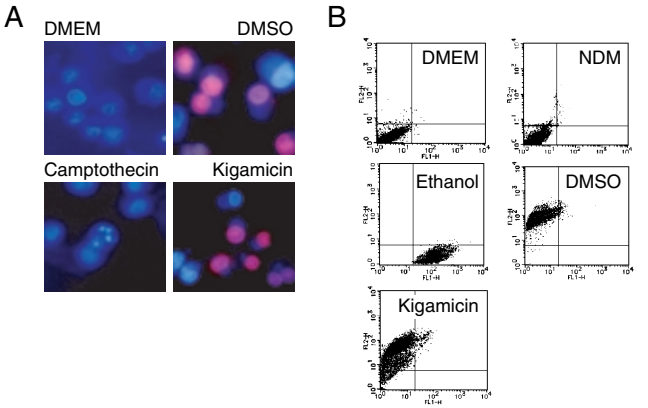

Fig. 3. Necrosis induced by kigamicin D. A. PANC-1 cells (1×10^6) were seeded in 10 cm dishes. After incubation for 24 h in nutrient-deprived medium with 0.1 μg/ml kigamicin D, or in DMEM with 10% DMSO or 200 n*M* camptothecin, propidium iodide and Hoechst staining was performed, and photos of the cells were taken. B. After exposure to 0.1 μg/ml kigamicin D for 6 h, both floating cells and attached cells were stained with annexin V and propidium iodide. Stained cells were analyzed using a fluorescence-activated cell sorter (Caliber, Becton Dickinson). PANC-1 cells exposed to 5% ethanol or to 10% DMSO were used as the control.

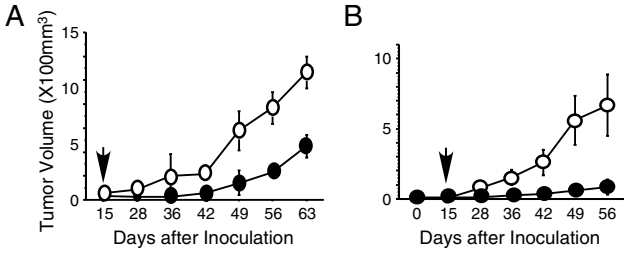

Fig. 4. Antitumor effect of kigamicin D on PANC-1 cells in nude mice. Female, 5-week-old BALB/cAn Ncrj-*nu*/*nu* mice were subcutaneously injected in the right side with 5×10^6 PANC-1 cells. Fifteen days later, the mice bearing tumors of around 5 mm in diameter were randomly assigned to the treatment group and vehicle group. The animals were subcutaneously injected with kigamicin D (3 μg) solution or vehicle (A), or orally force-fed with kigamicin D (15 μg) solution or vehicle (B) 6 days/week as described in "Materials and Methods." The tumors were measured with caliper weekly. The results are shown as means±SD. ▼ at the start of the treatment, ● the treatment group, ○ the vehicle group.

angiogenic ability of the tumor,[17] we examined tumor angiogenesis by immunohistochemical staining using anti-CD31 antibody in tumors treated with kigamicin D in comparison with that in untreated tumors.[18-22] After immunohistochemistry with CD-31 antibody, the microvessel density was calculated according to the reported method. No significant difference in microvessel density was observed between the tumors in the treatment group and the control group (MVD: control 196.9±38.9 vessels/mm^2; treated 210.4±32.3 vessels/mm^2) (Fig. 6).

To investigate the toxicity of kigamicin D, the esophagus, stomach, lungs, liver, and spleen were taken from the mice, and subjected to hematoxylin and eosin staining. No remarkable abnormality was observed (data not shown).

Kigamicin D inhibits the phosphorylation of Akt. In our previous study, we found that high expression of PKB/Akt was associated with cancer cells' tolerance to nutrient starvation.[9] Transfection with an antisense RNA expression vector for Akt significantly diminished the tolerance to nutrient deprivation. Therefore, we examined the effect of kigamicin D on PKB/Akt activation. Phosphorylation of Akt (Ser-473) was stimulated in PANC-1 cells exposed to nutrient-starved medium as previously observed,[9] and the addition of kigamicin D markedly inhibited the activation of Akt by the nutrient deprivation (Fig. 7A). Because we found that the presence of serum partly counteracted the protective effect of glucose (Fig. 2D), the effect of kigamicin D on the activation of Akt in the presence of both glucose and serum was examined. The result showed that the deprivation of amino acid also activated Akt, and kigamicin D blocked its activation regardless of the presence of glucose (Fig. 7A). This observation reinforced the hypothesis that blockade of Akt activation is responsible for kigamicin D's preferential cytotoxicity.

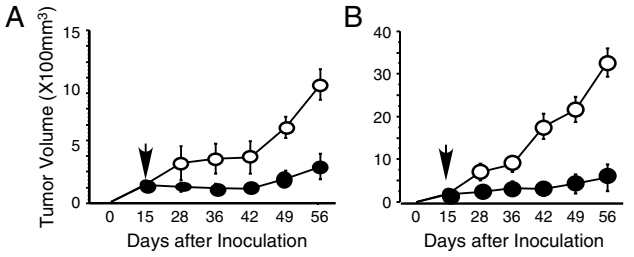

Fig. 5. Kigamicin D's effect on Capan-1 cells and MIA Paca-2 in nude mice. MIA Paca-2 (A) and Capan-1 (B) cells (5×10^6) were transplanted subcutaneously into nude mice. After the tumors had grown to around 5 mm in diameter, the mice were orally administered with kigamicin D (15 μg) solution or vehicle, 6 days/week. The tumors were measured weekly. ● the treatment group, ○ the vehicle group.

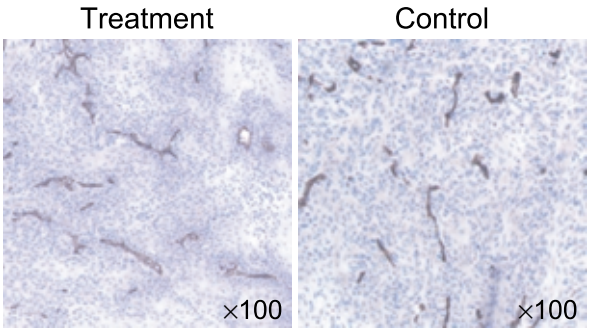

Fig. 6. Representative sections of subcutaneous tumors stained with CD31 antibody.

The effect of kigamicin D on Akt activation *in vivo* was also examined. The results in Fig. 7B clearly demonstrate that administration of kigamicin D inhibits the phosphorylation of Akt *in vivo* as well, strongly indicating that kigamicin D was absorbed and reached the tumor site, where it inhibited tumor development.

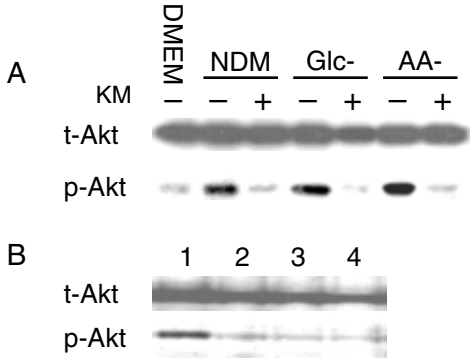

Fig. 7. Effect of kigamicin D on the phosphorylation of Akt (Ser-473). A. Kigamicin D inhibits the activation of Akt *in vitro*. PANC-1 cells were cultured in fresh DMEM media for 24 h, then washed with PBS, and the medium was changed to DMEM or nutrient-deprived medium or glucose-free medium or amino acids (AA)-free medium with or without 0.1 μg/ml kigamicin D. Cells were harvested 2 h later. A 10 μg aliquot of the cell lysate was loaded into each well for fractionation by 12% SDS-PAGE. Phosphorylation of Akt was investigated by western blot analysis with phospho-specific (Ser-473) Akt antibody (New England BioLabs). B. Activation of Akt in subcutaneous tumors. KM, kigamicin D; t-Akt, total Akt; p-Akt, phosphorylated Akt (Ser-473). Nude mice transplanted with PANC-1 cells had been given vehicle (lane 1) or 15 μg of kigamicin D orally every day for 6 weeks (lanes 2, 3), or only one oral administration of 15 μg of kigamicin D 2 h before sacrifice (lane 4). The tumors were then excised and immediately frozen with dry ice and subjected to western blot analysis.

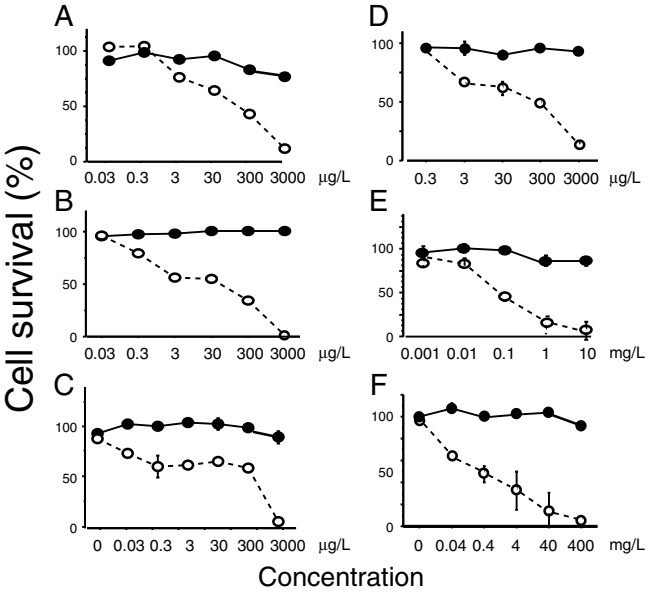

Fig. 8. Effect of conventional anticancer drugs on PANC-1 cells viability. Cells were seeded at a density of 1.0×10^4 cells/well in 96-well plates and incubated in normal medium DMEM (open circle), or nutrient-deprived medium (closed circle) for 24 h with or without the conventional chemotherapeutic agents, vincristine (A), 5-fluorouracil (B), taxol (C), doxorubicin (D), cisplatin (E) and campothecin (F) at the various concentrations indicated, and viable cells were counted 24 h later. The number of cells in the wells without anticancer drugs was set equal to 100%. The results are shown as means±SD.

Most conventional chemotherapeutic drugs showed weaker cytotoxicity to cancer cells under nutrient-deprived conditions. To examine the uniqueness of kigamicin D, we compared the anticancer effects of some representative conventional anticancer drugs, vincristine, 5-fluorouracil, taxol, doxorubicin, cisplatin, and camptothecin under nutrient-sufficient or -deprived conditions. As shown in Fig. 8, all the tested chemotherapeutic drugs killed PANC-1 cells in the nutrient-sufficient medium, but showed much weaker cytotoxicity in nutrient-deprived medium within 24 h. This result clearly demonstrated the difference between kigamicin D and conventional chemotherapeutic drugs.

Discussion

The process of tumor development is a balance between the integrals of cell production and cell loss. Malignant tumors are characterized by invasion, metastasis, and unrestrained growth, which have been reported to be sustained by the development of new blood vessels and a decrease in the number of cells undergoing apoptosis.[23] As a result, tumor cells induce the formation of a new blood supply from the preexisting vasculature, and this allows tumor cells to survive and proliferate.[24] Since angiogenesis is seldom activated in normal tissue, anticancer therapy targeting tumor angiogenesis is thought to be a promising strategy for treating cancer.[25-27] However, some recent reports have suggested that cancers may be able to elude the effect of angiogenesis inhibitors through genetic alterations that decrease the vascular dependence of tumor cells and thereby affect their therapeutic response to antiangiogenic therapy.[7]

There is also overwhelming evidence that, in spite of the activation of angiogenesis, cancer cells are still exposed to extreme insufficiency of nutrient supply due to the deformed construction of new vessels and the rapid proliferation of the cancer.[28, 29] Furthermore, some extremely hypovascular cancers, such as pancreatic cancers,[30] are among the most aggressive malignancies. They are capable of growing rapidly even in a nutrient-deficient environment and are insensitive to growth-inhibitory and apoptotic signals.[31] The question thus arises, how and where do pancreatic cancers obtain the nutrients needed to support their rapid proliferation? There must be mechanisms that facilitate the survival and proliferation of cancer cells in extreme adverse environments.

We previously discovered a hitherto unknown biological response of cancer cells to nutrient starvation, through which various cancer cells become tolerant to nutrient starvation, especially glucose starvation. Similar constitutive tolerance to nutrient deprivation has been observed in some poorly differentiated cancer cell lines, including pancreatic cancers, in which hypovascularity is commonly observed.[9, 10, 12] Based on our observations we hypothesized that the cancer cells might have acquired the ability to survive in an extremely adverse microenvironment not only through increased angiogenesis, but also by acquiring tolerance to nutrient starvation, i.e., by austerity.[10]

Based on these observations, we developed a screening method for compounds that might provide the basis for an anti-austerity strategy and identified kigamicin D, which has a novel structure and exhibits preferential cytotoxicity during nutrient starvation. Because nutrient deficiency seldom occurs in mature normal tissue, kigamicin D's anti-austerity ability should be specific to cancer cells. Indeed, in the present work, kigamicin D markedly inhibited tumor formation of cancer cells in nude mice, but did not show severe toxicity to normal tissues, indicating that our new anti-austerity strategy might be a feasible method for cancer therapy. Furthermore, very recently we also found that an anthelmintic, pyrvinium pamoate, which displays preferential cytotoxicity against PANC-1 cells during glucose starvation, also exhibits antitumor activity in nude mice (Esumi *et al*. to be published elsewhere). Taken together, all these findings indicate that anti-austerity is a novel and feasible strategy for cancer treatment.

The molecular and biochemical mechanisms of cancer cells' austerity and the pharmacological mechanisms of kigamicin D are not yet fully understood, but the inhibition of Akt phosphorylation by kigamicin D may have some relation with its preferential cytotoxicity. Recently we identified a new member of AMPK, ARK5, which is a cell survival factor, and showed that its activation regulated by Akt induces tolerance of cancer cells to nutrient starvation.[32] Thus, the Akt/ARK5 pathway is a new signaling pathway for the induction of cell survival that is closely associated with tumorigenesis. We also observed that activation of Akt/ARK5 can rescue cancer cells from necrosis induced by nutrient starvation, suggesting that Akt plays a critical role in the necrosis of cancer cells.[16] Therefore, the inhibition of Akt phosphorylation should, at least in part, be responsible for the preferential cytotoxicity of kigamicin D. On the other hand, an antisense RNA expression vector for Akt significantly diminished the tolerance of PANC-1 cells to nutrient starvation. However, wortmannin and LY294002 do not kill cancer cells under the same conditions, although Akt phosphorylation (Ser-473) was completely inhibited by these two compounds.[9] These results can not be explained easily. There seem to be two possibilities that might account for this discrepancy. One is that kigamicin D might have additional effects other than the inhibition of Akt phosphorylation. The other is that Akt activation at a site other than Ser-473 might be important in the austerity mechanisms. This needs further study.

Furthermore, we found that kigamicin D inhibited the activity of PI-3 kinase (to be published elsewhere). However, although wortmannin and LY294002 are representative PI-3 kinase inhibitors, wortmannin did not show cytotoxicity to cancer cells under nutrient starvation, while LY294002 showed high cytotoxicity, but only under conditions of amino acid-deprivation.[9] The observations suggest that the PI-3 kinase/Akt pathway might not be sufficient to explain the preferential cytotoxicity of kigamicin D. Some pathway other than PI-3 kinase/Akt might be involved. Another possibility is that phosphorylation at Ser-473 of Akt might not reflect its activity in austerity. These possibilities remain to be examined.

Interestingly, in our present study, we found that most conventional chemotherapeutic drugs showed markedly weaker cytotoxicity to cancer cells in nutrient-deprived medium. It is known that nutrient deprivation causes a delay in cell cycle progression and often results in G1 arrest.[33] It is not unexpected that these anticancer drugs show reduced anticancer activity under these conditions. It has also been reported that accumulation of heat shock proteins (HSPs) induced by prolonged energy deprivation in stationary tumor cells can be endogenous resistance factors to anticancer treatments.[34] Another possibility is that an anti-apoptotic pathway is activated in PANC-1 cells when they are subjected to nutrient starvation.[16, 35] The mechanisms of the loss of anticancer activity may be different among different drugs. The precise mechanism still needs to be elucidated, but it is reasonable to assume that kigamicin D is a different type of anticancer drug.

In conclusion, our present study has clearly demonstrated that cancer cells' tolerance to nutrient starvation, i.e., austerity, is a novel and feasible target for cancer therapy. Theoretically, it seems likely that combinations of anti-austerity agents and antiangiogenesis agents will have a powerful anticancer effect on human cancers, and this possibility should be pursued.

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