

## Antitumor Effect of Kigamicin D on Mouse Tumor Models

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**Abstract** Kigamicin D is a novel anticancer agent that was identified using a new screening strategy that targets the tolerance of cancer cells to nutrient starvation [1, 2]. Oral administration of kigamicin D was previously described to show a strong antitumor effect in human tumor xenograft models of pancreatic tumors [2]. In this paper we describe that kigamicin D shows the same selective cytotoxicity against normal human cells such as lung fibroblast and prostate stromal cells under nutrient starved condition as against cancer cells. Kigamicin D inhibited tumor cell-induced angiogenesis in a dorsal air sac assay. On the basis of these results we tested other human tumor xenograft models and transplantable syngeneic tumor models in order to determine the spectrum of activity of kigamicin D against various cancers. Kigamicin D showed a weak antitumor effect against LX-1 and DMS-273 lung cancers, but had no effect on DLD-1 colon cancers. When tested against syngeneic tumors, kigamicin D showed a weak antitumor effect against colon26, but showed augmentation of tumor growth on IMC carcinoma at a broad dosage level. Kigamicin D does not show good antitumor activity against human xenograft tumors except pancreatic tumors and murine syngeneic tumors. We found that kigamicin D has excellent antitumor effect specific to pancreatic cancers. Surprisingly, high dosage of kigamicin D increased tumor growth of IMC carcinoma by than 200%. The phenomenon suggests that kigamicin D may cause some immunological response to the tumor.

**Keywords** kigamicin D, xenograft, syngeneic, IMC-carcinoma

### Introduction

Human xenograft tumor models implanted in nude or scid mice have provided good insights for the therapeutic possibility of antitumor agents. In human pancreatic tumor xenograft models, tumors are thought to keep the natural characteristics of pancreatic cancers such as hypovascularity and aggressive malignant behavior despite poor blood supply. Kigamicin D showed strong antitumor effect on human tumor xenograft models of pancreatic tumors such as PANC-1, Capan-1, and MIA Paca-2 in nude or scid mice [2]. Kigamicin D also showed cytotoxicity selective to nutrient starved culture in normal cells such as fibroblasts and human prostate stromal cells as described below. The center of solid tumors is known to be hypoxic and deficient in nutrient because of it being avascular. It has been evidenced that PI3K promotes angiogenesis, and, PI3K inhibitors such as LY294002 and wortmannin have antiangiogenic activity *in vivo* [5, 6]. Kigamicin D was previously described to inhibit PI3K/Akt pathway and shows antiangiogenic activity [2]. These two findings, namely the cells' response to kigamicin D under nutrient starvation and the antiangiogenesis activity of kigamicin D, prompted us to assess the antitumor spectrum of kigamicin D. We studied human tumor xenograft models other than pancreatic tumors and transplantable syngeneic tumor

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models. Kigamicin D did not show good antitumor activity in these models unlike in human pancreatic xenografts. Antitumor activity of kigamicin D is considered to be specific to pancreatic tumors, possible because of its hypovascularity.

## Materials and Methods

### Preparation of Kigamicin D

Kigamicin D was prepared by large-scale production and the purification method was modified from that previously described [1] as follows. The strain *Amycolatopsis* sp. ML630-mF1 was inoculated into two 30-liter jar fermenters containing 15 liters of the culture medium per jar as previously described [3]. The fermentation was performed at 30°C at 6 days. The culture filtrate (24 liters) was applied to a Diaion HP-20 column (Mitsubishi Chemical Co., Tokyo, 1 liter, 5.5×40.5 cm) and eluted with 50% acetone-water. The eluate was concentrated to remove acetone and adjusted to pH 2.0 and extracted with an equivalent volume of butylacetate. After removal of butylacetate by evaporation and drying under vacuum, the dried paste (4.64 g) was charged on a silica gel column (Wako gel 200, Wako Pure Chemical Industries, Osaka, 206 g, 6×14 cm), and eluted stepwise with mixtures of CHCl<sub>3</sub> and methanol (50 : 1, 25 : 1, and 10 : 1). The active eluate was charged onto a reverse phase ODS column (Senshu Scientific Co. Ltd., Tokyo, ODS7515-12, 480 ml, 6×16 cm) and developed with a mixture of 40% acetonitrile and 60% water. Kigamicin A, C, D and E were separately eluted in this order. The yield and purity of each Kigamicin was as follows: Kigamicin D, 2,240 mg of 99.9% purity by HPLC analysis; Kigamicin A, 60 mg crystals; Kigamicin C, 800 mg of 87% purity; and Kigamicin E, 740 mg of 87% purity. Kigamicin D was very more effectively produced in jar fermenters than in flask cultures and it was easily purified without using HPLC.

Kigamicin D was dissolved and diluted with DMSO for *in vitro* assay.

For oral administration, kigamicin D was dissolved at the highest concentration administered in saline solution supplemented with 10% DMSO and 0.5% Tween 80, and stored in a refrigerator up to a week. Before administration the stock solution was diluted in saline to give the needed concentration in a 0.25 ml solution.

### Cells and Culture

Human cancer cell lines, PANC-1 (pancreatic cancer) and DLD-1 (colon cancer), and DMS-273 (lung cancers) were obtained from American Type Culture Collection

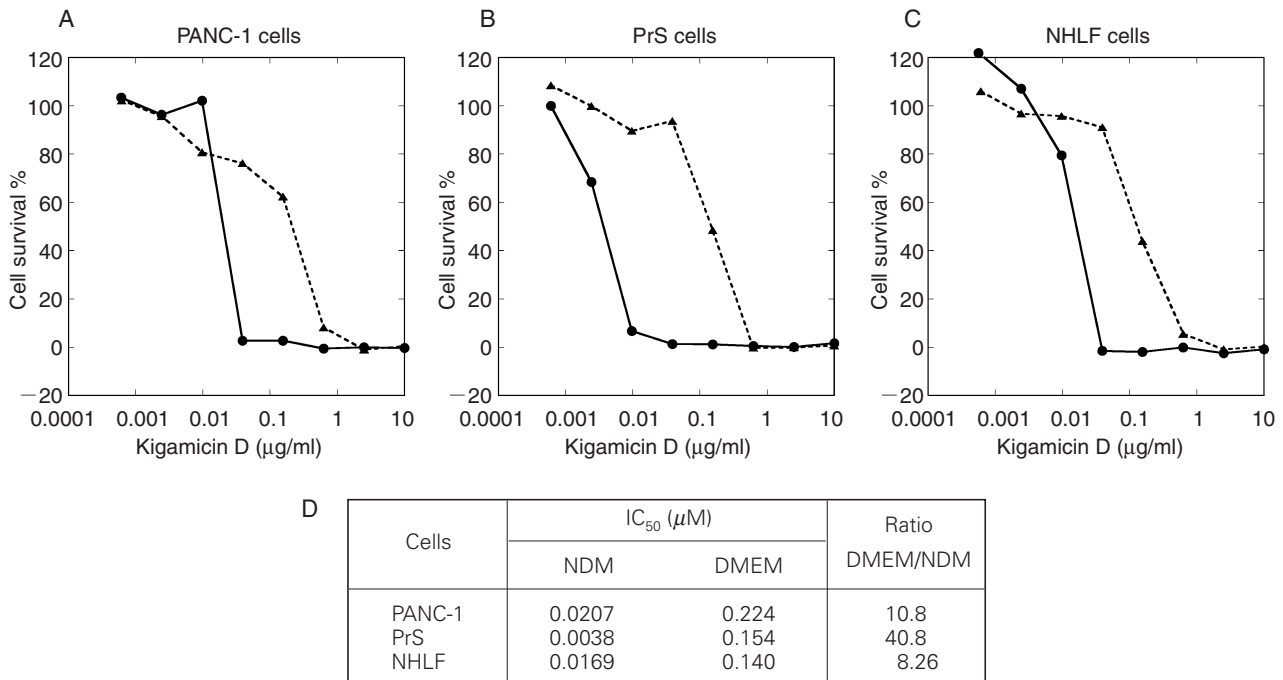
and European Collection of Cell Cultures, respectively. They were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 10% fetal bovine serum (FCS) (Tissue Culture Biologicals, CA, USA or Vitromex, Germany). LX-1 (lung cancer) was kindly supplied by Japanese Foundation for Cancer Research, Tokyo and maintained in RPMI1640 (Gibco, Invitrogen Co., CA, USA) supplemented with 10% FCS and 100 units/ml penicillin G, 100 µg/ml streptomycin. Murine colon 26 cells were maintained in RPMI1640 (Gibco, Invitrogen Co., CA, USA) supplemented with 10% FCS and 100 units/ml penicillin G, 100 µg/ml streptomycin. IMC carcinoma cells were maintained by a serial transplantation i.p. weekly in CDF<sub>1</sub> mice. Normal human lung fibroblast (NHLF) cells and normal human prostate stromal (PrS) cells were from BioWhittaker Inc. (Walkerville, MD, USA). NHLF was maintained in FGM-2 BulletKit (BioWhittaker Inc. (Walkerville, MD, USA). PrSC was maintained in DMEM supplemented with 10% FBS, 100 units/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 basic-FGF.

### *In Vitro* Assay of Selective Cytotoxicity under Nutrient-deprived Condition

Killing activity against PANC-1 cells under a nutrient starved condition was determined by comparing cell survival after 24 hours incubation in nutrient deprived medium (NDM) with cell survival in DMEM - 10% FCS culture as previously described [1, 2]. NDM was composed of only electrolytes and vitamins found in the composition of DMEM as following: CaCl<sub>2</sub>(2H<sub>2</sub>O), 265 mg/ml; Fe (NO<sub>3</sub>)<sub>3</sub>(H<sub>2</sub>O), 0.1 mg/liter; KCl, 400 mg/ml; MgSO<sub>4</sub>(7H<sub>2</sub>O), 200 mg/liter; NaCl, 6400 mg/liter; NaHCO<sub>3</sub>, 700 mg/liter; NaHPO<sub>4</sub>, 125 mg/liter; phenol red, 15 mg/liter; 25 mM HEPES buffer pH 7.4; and MEM vitamin solution (Life technologies, Inc., Rockville, MD). Less than 1 µl of kigamicin D solution or DMSO was added to each culture of 100 µl. After 24 hours treatment cell viability was evaluated by incubation for 3 hours with DMEM containing 10% Alamar Blue solution (Serotec Ltd., UK) [3] after PBS washing and measurement of fluorescence at 590 nm excited by 544 nm using Labsystems Fluoroscan II (Dainippon Seiyaku, Osaka).

### Angiogenesis

Tumor cell-induced angiogenesis in a dorsal air sac (DAS) assay was performed in mice to examine the effect of kigamicin D on the angiogenic response triggered by S-180 cells, according to the method described by Oikawa *et al.* [4]. Briefly, both sides of a Millipore ring were covered



**Fig. 1** Effects of kigamicin D on PANC-1 and normal (PrS and NHLF) cells.

PANC-1 (A), PrS (B) and NHLF (C) cells were treated by kigamicin D for 24 hours under DMEM-10% FCS (DMEM, broken lines) or nutrient deprived medium (NDM, solid lines). Cell survival after 24 hours treatment was determined by Alamar Blue assay. IC<sub>50</sub> values given were summarized in D.

with Millipore filters of 0.45- $\mu$ m pore size, and the resulting Millipore chamber was filled with a suspension of S-180 cells ( $2 \times 10^7$  cells) in 0.15 ml of PBS. The chamber containing S-180 cells was implanted into the preformed air sac in the dorsum of anesthetized (50 mg/kg pentobarbital, i.p) female ICR mice. The animals were divided into six groups composed of 6 mice for each group. Drugs were orally administered from day 1 to day 4. On day 5, the implanted chambers were removed from the subcutaneous fascia of treated mice, and then black rings of the same inner diameter as the Millipore rings were placed on skins at the sites exposed to direct contact with the chamber. The angiogenic response was assessed with dissecting microscope photographs by determining the number of newly formed blood vessels that were more than 3 mm in length within the area marked by the black ring.

#### Antitumor Activity *In Vivo*

Female BALB/c nu/nu (-), BALB/c and CDF<sub>1</sub> mice were purchased from Charles River Japan, Inc., Yokohama and maintained in a specific pathogen-free barrier facility, and provided with sterile food ad water *ad libitum*. Tumor cells were washed and suspended with Hank's balanced salt solution (Gibco, Invitrogen Co., CA, USA). Six weeks old

mice were transplanted with 0.1 ml of the tumor cells subcutaneously at groin as described in detail in the legends for each experiment. Kigamicin D was administered 5 days/week by oral administration after tumor grew around 6 mm diameters. Antitumor activity was monitored by measuring tumor volume and determined by tumor weight on the last day of each experiment. Tumor volume was calculated as follows: tumor volume (mm<sup>3</sup>)=length (mm) $\times$ width (mm)<sup>2</sup> $\times$ 0.5. The ratio of mean tumor weight of treated group to that of control group (T/C%) was calculated.

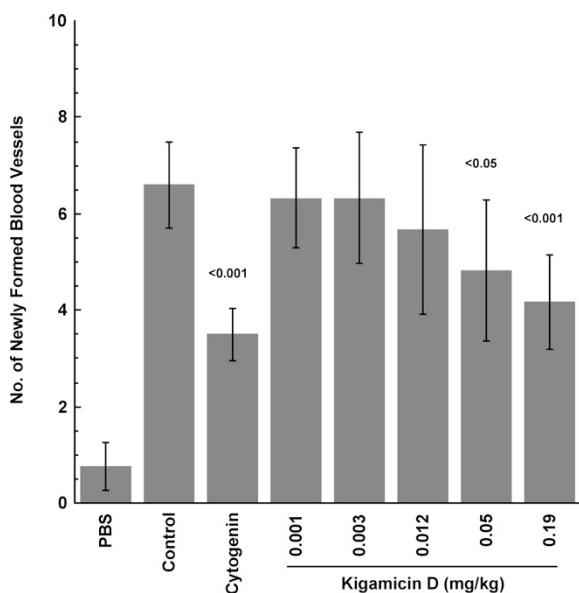
#### Statistical Analysis

All results were expressed as means $\pm$ S.D. 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 and Discussion

#### Effect on Normal Cells *In Vitro*

As shown in Fig. 1, kigamicin D showed selective cytotoxicity against nutrient-starved normal human cells



**Fig. 2** Inhibitory activity of kigamicin D on the angiogenic response induced by S-180 cells.

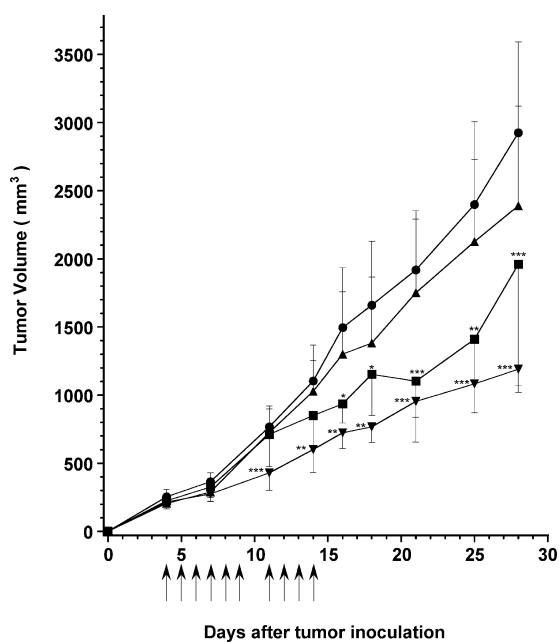
PBS and the other groups were implanted with a chamber containing PBS alone, and S-180 cells ( $2 \times 10^7$  cells/chamber), respectively. Kigamicin D (0.001, 0.003, 0.012, 0.05, and 0.19 mg/kg) and cytotogenin (100 mg/kg) as a positive control were orally administered on day 1, 2, 3 and 4. On day 5 newly formed blood vessels were evaluated as described "Materials and Methods". *P* values were calculated for Control.

such as lung fibroblast and prostate stromal cells. This cytotoxic effect is similar to that observed with PANC-1 cells.

These results led to the hypotheses that kigamicin D may show effect on other solid tumor beyond pancreatic cancers because nutrient deprivation and avascular tissue is commonly found in many tumors types.

### Inhibition of Angiogenesis

Kigamicin D inhibits PI3K/Akt pathway as previously described [2]. It has been shown that PI3K promotes angiogenesis, and LY294002 and wortmannin, PI3K inhibitors, have antiangiogenic activity *in vivo* [5, 6]. Effect of kigamicin D on angiogenesis was determined using DAS assay. As shown in Fig. 2, kigamicin D suppressed S-180 cell-induced angiogenesis in a dose dependent manner. In human pancreatic tumors kigamicin D did not inhibit tumor angiogenesis by immunohistochemical staining using anti-CD30 antibody [2]. Growth of solid tumors depends on angiogenesis, the process by which new blood vessels develop from the endothelium of a pre-existing vasculature. Many key players have been identified, but basic mechanisms have been unclear. Various mechanisms may work all



**Fig. 3** Effect of kigamicin D on LX-1 tumor in nude mice.

BALB/c nu/nu female mice were inoculated subcutaneously with  $5 \times 10^6$  human small cell lung carcinoma LX-1 cells on day 0. Kigamicin D was administered orally daily from day 4 to day 14 except day 10. As a positive control, adriamycin (2.5 mg/kg) was administered intraperitoneally according to the same schedule.

●, Control (none); ▲, kigamicin 0.75 mg/kg; ■, kigamicin 0.19 mg/kg; ▼, adriamycin 2.5 mg/kg; \**p*<0.05; \*\**p*<0.01; \*\*\**p*<0.001; vs. control.

together in the same tumor or may be selectively involved in a specific tumor type or host environment. Based on the similar effect of kigamicin D on normal cells and pancreatic cells and inhibition of angiogenesis by kigamicin D we tested the effect of kigamicin D on various kinds of tumor models.

### Human Xenograft Models

Human lung cancer cells LX-1 and DMS-273 were transplanted into the inguinal area of mice which were treated by the oral administration of kigamicin D. As shown in Fig. 3 and Table 1 administration of 0.75 mg/kg of kigamicin D showed significant effect on LX-1 tumor by T/C% of 61.3%, but 0.19 mg/kg was not effective. As shown in Table 2, 0.75 mg/kg of kigamicin D significantly suppressed the growth of DMS-273 tumor by T/C% of 45.3%, but 0.05 and 0.19 mg/kg showed slight inhibition which was not significant. Against DLD-1 colon cancers kigamicin D had no effect (data not shown.). On the whole, kigamicin D did not have good antitumor activity to these models unlike the activity in human pancreatic xenografts as previously described [2].

**Table 1** Antitumor effect of kigamicin D on LX-1 tumor *in vivo*

Drug	Dose mg/kg	Tumor weight (mg) mean±S.D.	T/C %	P
None	0	2394±397	100	
Kigamicin D	0.19	2049±541	85.6	n.s.
	0.75	1468±363	61.3	<0.01
Adriamycin	2.5	1190±366	49.7	<0.001

Detail was described in the legend of Fig. 3. On day 28 tumors were removed from mice and tumor weights were measured.

**Table 2** Antitumor effect of kigamicin D on DMS-273 tumor *in vivo*

Drug	Dose mg/kg	Tumor weight (mg) mean±S.D.	T/C %	P
None	0	2593±600	100	
Kigamicin D	0.05	2202±609	84.9	n.s.
	0.19	1825±792	70.4	n.s.
	0.75	1176±925	45.3	<0.05
Adriamycin	2.5	1170±813	45.1	n.s.

BALB/c nu/nu female mice were inoculated subcutaneously with  $5 \times 10^6$  human small cell lung carcinoma DMS-273 cells on day 0. Kigamicin D was administered orally 5 days/week from day 14 to day 32. As a positive control adriamycin (2.5 mg/kg) was administered intraperitoneally on day 14, 16, 18, 20, 22, 24, and 26. On day 35, tumors were removed from mice and tumor weights were measured.

### Syngeneic Tumor Models

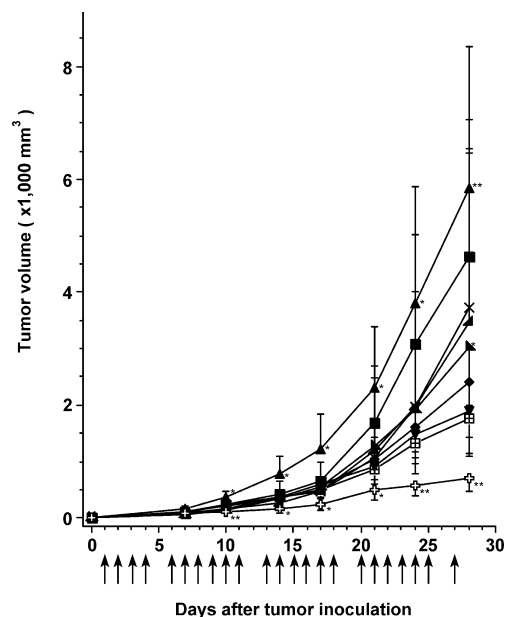
We have observed that kigamicin D strongly increased cellular immunity [5]. In order to study a relationship between antitumor effect and immunomodulating activity of kigamicin D, antitumor activity was tested using some syngeneic tumor models. Effect of kigamicin D on murine colon26 carcinoma cells was shown in Table 3. It had no effect against colon 26 tumor. Against IMC carcinoma cells, sensitive to immunomodulator such as bestatin, a broad dosage range from 0.003 to 12 mg/kg of kigamicin D increased tumor weight as shown in Fig. 4 and Table 4. The relationship between dosage and tumor growth of IMC carcinoma was an inverted bell-shaped curve. T/C% of 3, and 12 mg/kg were more than 200%.

Kigamicin D showed a strong antitumor effect on human tumor xenograft models of pancreatic tumors as previously described [2]. But it does not show good antitumor activity in a broad spectrum of human xenograft tumors and murine syngeneic tumors as described in this paper. Kigamicin

**Table 3** Antitumor effect of kigamicin D on murine colon 26 tumor *in vivo*

Drug	Dose mg/kg	Tumor weight (mg) mean±S.D.	T/C %	P
None	0	7620±2400	100	
Kigamicin D	0.012	5645±4161	74	n.s.
	0.05	7967±1847	105	n.s.
	0.19	5394±2506	70.8	n.s.
	0.75	7755±3202	102	n.s.
Furtulon	30	2221±1928	29.1	<0.01

BALB/c female mice were inoculated subcutaneously with  $2 \times 10^6$  colon 26 cells on day 0. Kigamicin D was administered orally 5 days/week from day 1 to day 34. As a positive control, furtulon (5' DFUR, a precursor of 5-FU converted by thymidine phosphorylase, 30 mg/kg) was administered orally by the same schedule as kigamicin D. On day 35, tumors were removed from mice and tumor weights were measured.

**Fig. 4** Effect of kigamicin D on IMC carcinoma.

CDF<sub>1</sub> female mice were inoculated subcutaneously with  $10^6$  IMC carcinoma cells on day 0. Kigamicin D was administered orally 5 days/week from day 1 to day 27. As a positive control, cyclophosphamide (100 mg/kg) was administered intraperitoneally on day 1, 4, 8, 11, 15, 18, 22, and 25. P values were calculated for Control.

□, Control (none); ▲, kigamicin D 12 mg/kg; ■, kigamicin D 3 mg/kg; ◆, kigamicin D 0.75 mg/kg; ▼, kigamicin D 0.19 mg/kg; ▲, kigamicin D 0.05 mg/kg; ▽, kigamicin D 0.012 mg/kg; ×, kigamicin D 0.003 mg/kg; ⊕, CY 100 mg/kg; \*p<0.05; \*\*p<0.01; vs. control.

**Table 4** Antitumor effect of kigamicin D on murine IMC carcinoma *in vivo*

Drug	Dose mg/kg	Tumor weight (mg) mean±S.D.	T/C %	P
None	0	2022±762	100	
Kigamicin D	0.003	3266±1833	162	n.s.
	0.012	2888±635	143	<0.05
	0.05	2893±1852	143	n.s.
	0.19	2263±593	112	n.s.
	0.75	2553±879	126	n.s.
	3	4049±1744	200	<0.05
	12	4677±1361	231	<0.01
Cyclophosphamide				
	100	740±211	36.6	<0.01

Detail was described in the legend of Fig. 4. As a positive control, cyclophosphamide (100 mg/kg) was administered intraperitoneally on day 1, 4, 8, 11, 15, 18, 22, and 25. On day 28, tumors were removed from mice and tumor weights were measured.

D has excellent antitumor effect specific to pancreatic cancers, which may be related to pancreatic cancer-specific microenvironment such as extreme hypovascularity, hypoxia, *etc.*

The augmentation of tumor growth of IMC carcinoma by kigamicin D may be brought about by immunosuppressive effect or immunological tolerance to the tumor cells. As described elsewhere [5], kigamicin D did not show immunosuppressive activity and any effect on NK cells activity, but showed remarkable increase of cellular immunity. Kigamicin D was thought to act on antigen presenting cells such as dendritic cells and macrophages. The activity on immature dendritic cells may bring about immunological tolerance and the increase of the growth of IMC carcinoma.

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