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Kigamicins, Novel Antitumor Antibiotics

I. Taxonomy, Isolation, Physico-chemical Properties and Biological Activities

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Novel antibiotics named kigamicin A, B, C, D, and E were discovered from the culture broth of *Amycolatopsis* sp. ML630-mF1 by their selective killing activity against PANC-1 cells only under a nutrient starved condition. Under a condition of nutrient starvation, kigamicins A, B, C, and D inhibited PANC-1 cell survival at 100 times lower concentration than in normal culture. Kigamicins showed antimicrobial activity against Gram-positive bacteria including methicillin resistant *Staphylococcus aureus* (MRSA). Kigamicin D inhibited the growth of various mouse tumor cell lines at IC_{50} of about 1 μg/ml.

Tolerance to nutrient deprivation as well as angiogenesis is essential for malignant tumor progression because the tumor microenvironment is characterized by insufficient oxygen and nutrient supplies. Because elimination of the tolerance might serve as a new strategy for cancer therapy[1], we have screened culture broths of soil microorganisms for specific cytotoxic activity against PANC-1 cells under a nutrient starved condition. Pancreatic cell line, PANC-1 is known to be extremely resistant to glucose and amino acid starvation, whereas normal human fibroblasts die within 24 hours[1]. In the course of screening we found new antibiotics as shown in Fig. 1 and named these kigamicins after kiga, a Japanese word meaning starvation. Kigamicin D showed good therapeutic activity to PANC-1 cells implanted into nude mice, and the mechanism of the removal of tolerance of the tumor cells to nutrient deprivation by kigamicin D was described in the another paper[2]. Structure elucidation of the kigamicins is reported separately[3]. In this paper, the taxonomy of the producer, isolation, physico-chemical properties, and biological activities of the kigamicins are reported.

Materials and Methods

Screening and Assay of Kigamicins

Killing activity against PANC-1 cells only under a nutrient starved condition was determined by comparing cell survival after 24 hours incubation in nutrient deprived medium (NDM) as previously described[1,2]. NDM was composed of only electrolytes and vitamins according to the composition of DMEM as following: $CaCl_2(2H_2O)$, 265 mg/ml; $Fe(NO_3)_3(H_2O)$, 0.1 mg/liter; KCl, 400 mg/ml; $MgSO_4(7H_2O)$, 200 mg/liter; NaCl, 6400 mg/liter; $NaHCO_3$, 700 mg/liter; $NaHPO_4$, 125 mg/liter; phenol red, 15 mg/liter; 25 mM HEPES buffer pH 7.4; and MEM vitamin solution (Life technologies, Inc., Rockville, MD). After 24 hours treatment with broths or fractions containing kigamicins, cell viability was measured with WST-1 cell counting kit (Dojindo Co., Kumamoto, Japan). For purification of kigamicins color on TLC plates and UV adsorption curves obtained by HPLC with a differential refractometer were useful.

Microorganism

The kigamicin producing strain *Amycolatopsis* sp.

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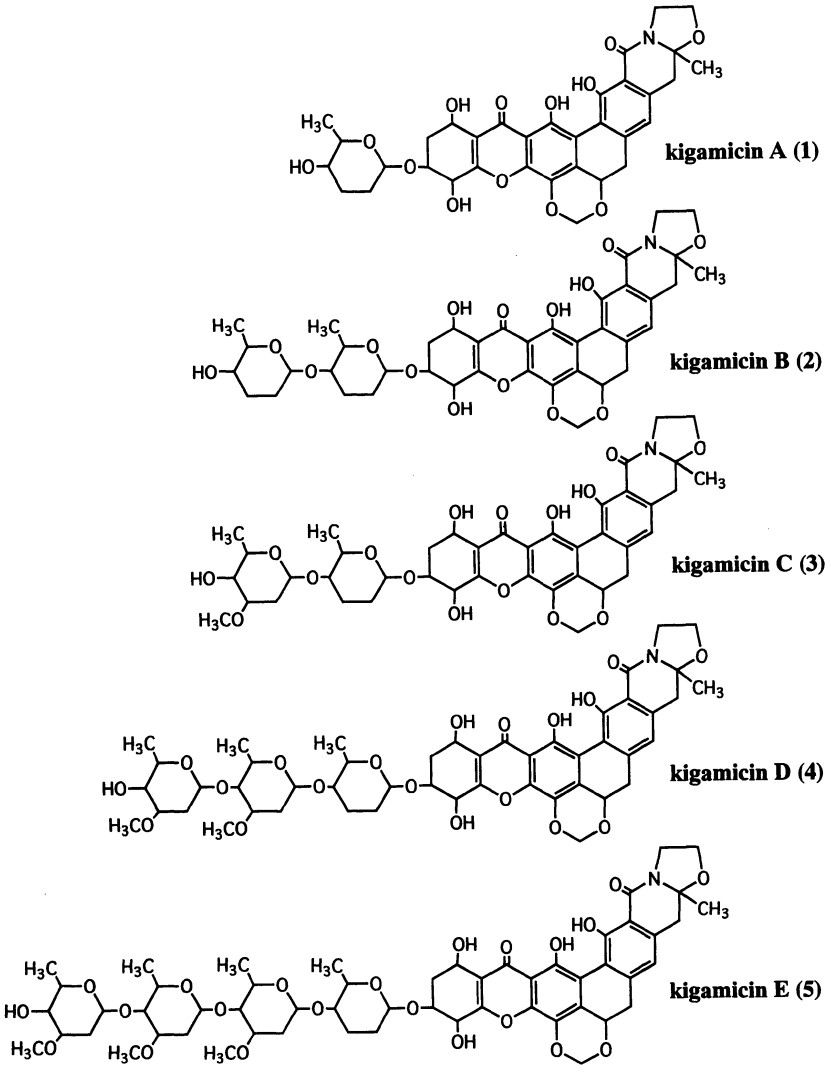
Fig. 1. Structure of kigamicins.



ML630-mF1 was isolated from a soil sample collected in the City of Toba, Mie prefecture, Japan. It was deposited in the National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan under the accession number FERM P-18875.

Taxonomic Studies

Morphological, cultural and physiological properties of the strain ML630-mF1 were examined according to the methods described by SHIRLING and GOTTLIEB[4], and WAKSMAN[5]. Detailed observation of mycelial morphologies was performed with the use of a scanning electron microscope (Model S-570, Hitachi) after strain ML630-MF1 was incubated on sucrose-nitrate agar at 27°C for 10 days. Cells used for chemotaxonomic analysis were obtained upon incubating the organism at 27°C for 4 days in yeast extract-glucose broth (1.0% yeast extract, 1.0% glucose, pH 7.2) on a rotary shaker. Whole cell hydrolysates were analyzed for diaminopimelic acid isomers using thin layer chromatography (TLC) according to the method of STANECK and ROBERTS[6]. Whole-cell sugars were prepared by the methods of LECHEVALIER and LECHEVALIER[7], and analyzed using TLC[8]. Phospholipids and mycolic acids were analyzed using TLC by the procedures of MINNIKIN *et al.*[9,10]. Menaquinones were extracted with the method of COLLINS *et al.*[11], and analyzed by LC-MS (model M-1200H, Hitachi) with a CAPCELL PAK C_{18} column (150 mm by 4.6 mm, Shiseido Fine Chemicals, Japan) using methanol-isopropanol (2 : 1, v/v) as the mobile phase.

A total DNA sample of strain ML630-mF1 was prepared as reported[12]. The 16S rDNA (16S ribosomal RNA gene, 1239 bp, positions 27~1290, *Escherichia coli* numbering system[13]) was amplified by polymerase chain reaction (PCR) using genomic DNA of strain ML630-mF1 and sequenced[14]. A search of the most related sequences was performed using the BLAST algorithm in the DNA Data Bank of Japan (DDBJ)[15], Mishima, Japan.

Fermentation

The strain *Amycolatopsis* sp. ML630-mF1 grown on a yeast-starch agar slant was inoculated into 500-ml Erlenmeyer flasks containing 110 ml of the medium [galactose 2.0%, dextrin 2.0%, Bactosoytone (Difco) 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%, silicon oil (Shin-Etsu Chemical Industry, KM-70) 0.03%, pH 7.4]. It was shake-cultured on a rotary shaker (180 rpm, 8 cm) at 30°C for 3 days. One ml of this seed culture was inoculated into 110 ml of production medium and cultured at 28°C for 4 days on a rotary shaker. The production medium was prepared by adding glycerol 1% to the seed culture medium.

Physico-chemical Properties

HRESI-MS spectra were measured with a JEOL JMS-T100LC spectrometer. FAB-MS spectra were measured with a JOEL JMS-SX102 spectrometer. Optical rotations were measured with a Perkin-Elmer 241 polarimeter using a microcell (light path 10 cm). Melting points were determined on a Yanagimoto apparatus and are uncorrected. UV and IR spectra were recorded on a Hitachi U-3210 spectrometer and a Horiba FT-210 spectrometer, respectively.

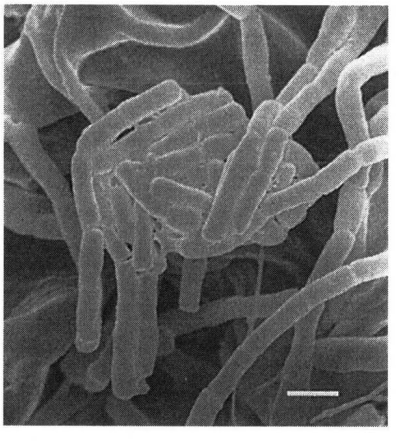
Results and Discussion

Taxonomic Features of Strain ML630-mF1

Strain ML630-mF1 produced well-branched substrate mycelia. This strain formed long aerial hyphae which were straight or flexous. Both substrate and aerial hyphae formed nocardioform fragmentation. The spore was cylindrical with smooth surface and 0.4~0.6×0.8~1.9 μm in size (Photo 1). No synnemata, sclerotia or sporangia were observed.

The cultural characteristics of strain ML630-mF1 on various agar media are shown in Table 1. The aerial mycelia were white. The substrate mycelia were pale yellow to pale yellowish brown. The soluble pigments were not produced.

Photo 1. Scanning electron micrograph of strain ML630-mF1 grown on sucrose-nitrate agar at 27°C for 10 days.



Bar=1 μm.

Table 1. Culture characteristics of strain ML630-mF1.

Medium	Growth	Aerial mycelium	Soluble pigment
sucrose-nitrate agar	Colorless	White	Absent
Yeast extract-malt extract agar (ISP med. No. 2)	Mustard (2le)~Cinnamon (3le)	White	Absent
Oatmeal agar (ISP med. No. 3)	Colorless~Bamboo (2 gc)	White	Absent
Inorganic salts-starch agar (ISP med. No. 4)	Colorless	White	Absent
Glycerol-asparagine agar (ISP med. No. 5)	Bamboo (2 gc)~Mustard (2 le)	White-Sand (3cb)	Absent
Tyrosine agar (ISP med. No. 7)	Bamboo (2 gc)~Mustard (2 le)	White-ivory Tint (2cb)	Absent

Physiological characteristics and carbohydrate utilizations are shown in Table 2. Permissive temperature ranges for growth of the strain were 20°C to 37°C. The optimal temperatures for growth of strain ML630-mF1 was near 30°C.

Whole-cell hydrolysates of strain ML630-mF1 contained *meso*-diaminopimelic acid, galactose and arabinose. Phospholipid pattern was type PII; phosphatidylethanolamine was present but none of phosphatidylcholine or unknown glucosamine-containing phospholipids was found. Mycolic acids were absent. Predominant menaquinones were MK-9(H_4).

The partial 16S rDNA sequence of strain ML630-mF1 showed high levels of identity with strains from the genus *Amycolatopsis*, such as *Amycolatopsis albidoflavus* AJ252832 (1225/1241, 98%), *A. rubidus* AF222022 (1200/1219, 98%), *A. mediterranei* AY083603 (1212/1239, 97%). *A. azurea* AJ400709 (1209/1238, 97%) and *A. coloradensis* AJ421142 (1204/1239, 97%)

These phenotypic and genotypic properties suggested that strain ML630-mF1 belonged to the genus *Amycolatopsis*[14]. Therefore, the strain was identified as *Amycolatopsis* sp. ML630-mF1.

Table 2. Physological characteristics of strain ML630-mF1.

Temparature range for growth	20~37°C
Optimum temparature	30°C
Formation of melanoid pigment	Negative
Hydrolysis of starch	Negative
Reduction of nitrate	Probably negative
Utilization of carbon sources	
Positive	D-xylose, D-glucose, D-fructose, *myo* -inositol, D-mannitol
Probably negative	L-arabinose, sucrose
Negative	L-rhamnose, raffinose

Fermentation and Isolation

The strain *Amycolatopsis* sp. ML630-mF1 was precultured in a 500-ml Erlenmeyer flask containing 110 ml

Table 3. Physico-chemical properties of kigamicins.

	A	B	C	D	E
Appearance	Yellow powder	Yellow powder	Yellow powder	Yellow powder	Yellow powder
FAB-MS (*m/z*)	666.3 $(M+H)^+$	780.4 $(M+H)^+$	810.4 $(M+H)^+$	954.6 $(M+H)^+$	1098.6 $(M+H)^+$
	664.3 $(M-H)^-$	778.4$(M-H)^-$	808.4 $(M-H)^-$	952.4 $(M-H)^-$	1096.6 $(M-H)^-$
HRESI-MS (m/z) Found	688.2055	802.2647	832.2763	976.3531	1120.4357
Calcd. $(M+Na)^+$	688.2006	802.2687	832.2793	976.3579	1120.4365
Molecular formula	$C_{34}H_{35}NO_{13}$	$C_{40}H_{45}NO_{15}$	$C_{41}H_{47}NO_{16}$	$C_{48}H_{59}NO_{19}$	$C_{55}H_{71}NO_{22}$
Optical rotation					
$[\alpha]^{24}$(c1.0, MeOH)	-153.0°		-154.0°	-190.6°	-175.2°
Mp (°C)	225-227	225-227	210-212	210-212	210-212
UV					
λ_{max}^{MeOH} nm (log ε)	217 (4.67),	217 (4.73),	217 (4.37),	217 (4.56),	217 (4.59),
	236 (4.64),	236 (4.71),	236 (4.34),	236 (4.52),	236 (4.55),
	254 (4.64),	254 (4.71),	254 (4.32),	254 (4.50),	254 (4.52),
	280 (4.54).	280 (4.61).	280 (4.22).	280 (4.39).	280 (4.42).
	341 (4.28)	341 (4.35)	341 (4.02)	341 (4.12)	341 (4.16)
$\lambda_{max}^{MeOH-HCl}$ nm (log ε)	217 (4.67),	217 (4.76),	217 (4.36),	217 (4.54),	217 (4.57),
	236 (4.67),	236 (4.74),	236 (4.34),	236 (4.51),	236 (4.55),
	254 (4.68),	254 (4.76),	254 (4.35),	254 (4.52),	254 (4.55),
	280 (4.57).	280 (4.64).	280 (4.25).	280 (4.41).	280 (4.22).
	341 (4.33)	341 (4.40)	341 (3.99)	341 (4.18)	341 (4.19)
$\lambda_{max}^{MeOH-NaOH}$ nm (log ε)	280 (4.49),	280 (4.57),	280 (4.15),	280 (4.32),	280 (4.35),
	366 (4.30)	366 (4.37)	366 (3.93)	366 (4.12)	366 (4.14)
IR (KBr) cm^{-1}	3530, 1620,	3450, 1617,	3440, 1620,	3450, 1620,	3450, 1620,
	1469, 1436,	1440, 1274,	1467, 1442,	1467, 1442,	1467, 1442,
	1257, 1064	1257, 1060	1276, 1062	1276, 1062	1276, 1062
HPLC (RT min)*	4.46	7.99	7.74	11.69	16.49

*HPLC: Capcell pak (TypeUG120Å 5μm, d4.6x150 mm, Shiseido Co.), developped by acetonitrile-water (40:60)

of medium described in the Materials and Methods on a rotary shaker at 30°C for 3 days. One ml of the cultured broth thus prepared was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of production medium. The culture flasks containing 12 liters of medium altogether were shaken at 27°C for 4 days.

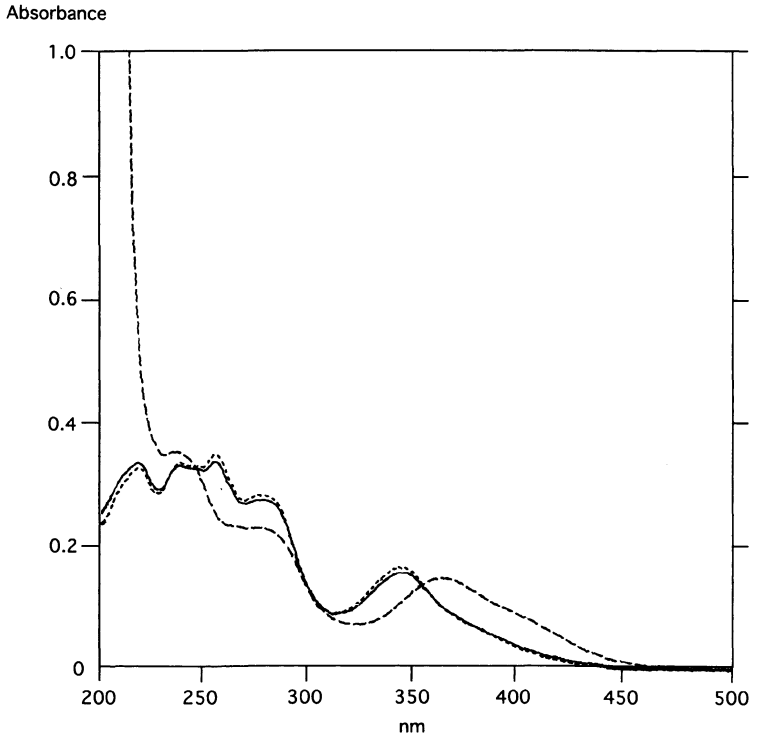
The culture filtrate (10,270 ml) was adjusted to pH 2.0 and extracted with butylacetate. Dried paste (1.59 g) was charged on a silica gel column (Merck silica gel 60, 240 g), and eluted stepwise with mixtures of $CHCl_3$ and methanol (50 : 1, 25 : 1, and 10 : 1). Active eluate was separated into two parts, the first eluate containing 325.2 mg material followed by a second fraction containing 256.2 mg. Each eluate was charged onto a reverse phase ODS column (Senshu Scintific Co. Ltd. ODS7515-12, 60 g) and developed with a mixture of acetonitrile and water. From the first eluate three active fractions (41.9 mg, 107.9 mg, and 30.1 mg) containing kigamicins C, D, and E, respectively as the main components were separated by developing with 40% acetonitrile - 60% water, respectively. Each fraction was further purified by chromatography using reverse phase HPLC (Shiseido, Capcell Pak ODS UG120, 30×250 mm) with a solvent of 40% actonitrile - 60% water. Thus kigamicin C (31.6 mg), kigamicin D (85.3 mg), and kigamicin E (19.4 mg) were purified as yellow powders. The second eluate from the silica gel column chromatography was applied on reverse phase ODS column. Kigamicin A was eluted with 30% acetonitrile - 70% water, and crystallized as plates (25.8 mg) from the condensation.

Kigamicins B (4.1 mg) was purified from another culture (3 liters) by almost the same purification steps along with kigamicin C (14.9 mg), D (46.6 mg), and E (21.8 mg).

Physico-chemical Properties

Physico-chemical properties of kigamicins A, B, C, D, and E are shown in Table 3. Kigamicins has characteristic UV spectra as shown in Fig. 2. All compounds are soluble in $CHCl_3$, EtOAc, MeOH and DMSO, but hardly soluble in water. The molecular formula of kigamicins was established by field desorption mass spectroscopy and high resolution mass spectrometry.

Fig. 2. UV spectra of kigamicin D.



UV spectra of kigamicin D (10 μg/ml) were recorded in MeOH, 0.01 N HCl-MeOH, or 0.01 N NaOH-MeOH solution as shown in solid, dotted, and dashed lines, respectively.

Table 4. Antimicrobial activities of kigamicins.

Organisms	MIC (μg/ml)				
	A	B	C	D	E
Staphylococcus aureus FDA209P	0.05	0.10	0.05	0.20	0.20
S. aureus Smith	<0.025	0.10	0.05	<0.025	0.025
S. aureus MS9610	0.05	0.20	0.10	0.39	0.39
S. aureus MRSA No.5	0.10	0.20	0.20	0.39	0.78
S. aureus MRSA No.17	0.10	0.20	0.20	0.39	0.78
S. aureus MS16526 (MRSA)	0.20	0.20	0.20	0.39	0.78
S. aureus TY-04282 (MRSA)	0.39	0.20	0.05	0.39	0.39
Micococcus luteus FDA16	0.10	0.20	0.10	0.39	0.39
M. luteus IFO03333	0.10	0.20	0.10	0.39	0.39
M. luteus PCI1001	0.05	0.20	0.20	0.39	0.78
B. subtilis NRRL B-558	<0.025	<0.025	0.10	0.39	<0.025
B. subtilis PCI219	<0.025	<0.025	0.05	0.10	<0.025
B. cereus ATCC10702	<0.025	<0.025	0.05	0.20	0.20
Corynebacterium bovis 1810	0.05	0.20	0.10	0.20	0.39
Escherichi coli NIHJ	100	>100	100	>100	>100
E. coli K-12	>100	>100	100	>100	>100
E. coli K-12 ML1629	>50	>100	100	100	100
E. coli BEM11	>50	>100	100	>100	>100
E. coli BE1121	1.56	>100	100	>100	>100
E. coli BE1186	>50	>100	>100	>100	>100
Shigella dysenteriae JS11910	>50	>50	>50	>50	>50
Sh. Flexneri 4b JS11811	>50	>50	>50	>50	>50
Sh. Sonnei JS11746	>100	>100	100	>100	>100
Salmonella enteritidis 1891	>50	>50	>50	>50	>50
Proteus vulgaris OX19	100	>100	>100	>100	>100
P. mirabilis IFM OM-9	100	>100	>50	>100	>100
Providencia rettgeri GN311	>50	>50	>50	>100	>50
Prov. rettgeri GN466	>50	>50	>50	>50	>50
Serratia marcescens	>50	>50	>50	>50	>50
Pseudomonas aeruginosa A3	>50	>50	>50	>50	>50
Ps.aerug. GN315	>50	>50	>100	>50	>50
Klebsiella pneumoniae PCI602	>50	>50	>50	>50	>50
Mycobacterium segamatis ATCC607	>50	>50	>100	50	>50
Candida albicans 3147	>50	1.56	3.13	3.13	12.5

Biological Activities

The antimicrobial activities of kigamicins A, B, C, D, and E are shown in Table 4. They inhibit the growth of Gram-positive bacteria including *Staphylococcus aureus* MRSA, but are not active against Gram-negative bacteria.

The selective toxicity of kigamicins against PANC-1 cells under a nutrient starved condition is shown in Fig. 3. Kigamicins A, B, C, D, and E inhibited PANC-1 cell survival at concentrations 100 times lower under a nutrient starved condition than in normal culture. Kigamicin E showed less selective toxicity.

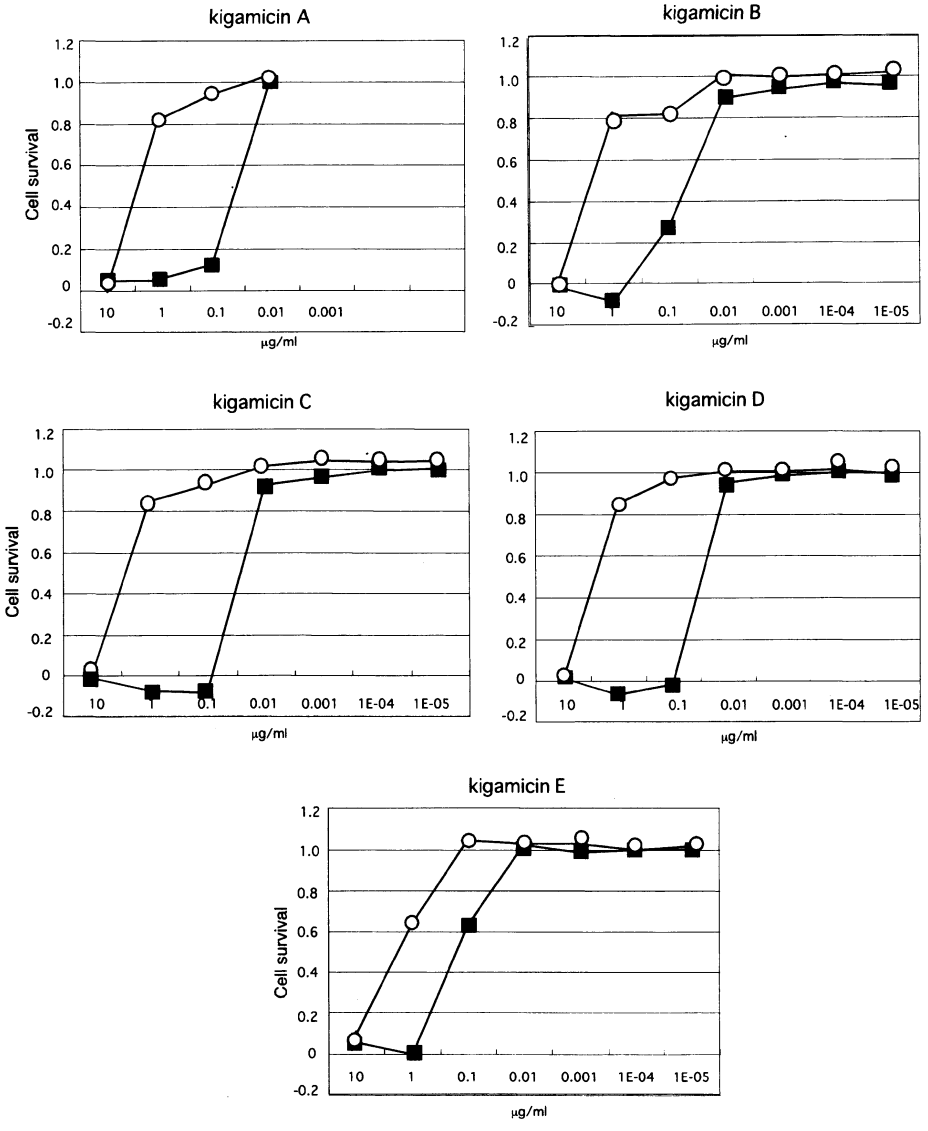
The inhibitory effect of kigamicin D on the growth of various mouse tumor cell lines is shown in Table 5. Growth of all the cell lines was inhibited with similar dose-

Table 5. Effect of kigamicin D on the growth of mouse tumor cell lines.

Cell line	IC_{50} (μg/ml)
LB32T	0.50
L-1210	0.99
EL-4	0.92
P388D1	0.86
B16 BL6	1.01
FS3	1.01
Colon26	0.954

L-1210, EL-4, P388D1, B16 BL6, FS3, and Colon26 cells were cultured for 2 days. LB32T cells was cultured for 3 days. The cell growh was determined by MTT assay.

Fig. 3. Selective toxicity of kigamicins to PANC-1 cells under a nutrient starved condition.



Activity of killing PANC-1 cells by kigamicins was determined by measurring cell survival after 24 hours incubation in nutrient deprived medium (■) or in Dulbecco's modified Eagle's medium (○) as described in Materials and Methods.

dependent curves, and in each case the IC_{50} was about 1 μg/ml.

The mechanism of action of kigamicin is reported separately[2], where we describe that kigamicin D blocks the activation of PKB/Akt caused by withdrawal of nutrients from culture medium.

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