NEW ANTIVIRAL ANTIBIOTICS, KISTAMICINS A AND B

I. TAXONOMY, PRODUCTION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

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A new strain of *Microtetraspora parvosata* subsp. *kistnae* subsp. nov. (ATCC 55076) was found to produce new antiviral antibiotics, designated kistamicins A and B. These antibiotics exhibited activity against influenza virus type A and moderate activity against Gram-positive bacteria.

Chemoprevention of viruses causing mammalian diseases is becoming more and more important. In our continuing search for novel antiviral activities from microbial metabolites, a new actinomycete strain No. S382-8 isolated from a soil sample collected near the Kistna River in Andhra Pradesh State, India, was found to produce new antiviral antibiotics, designated kistamicins A and B (formerly called BU-4344V). These have potent activity against influenza virus type A Victoria strain in infected Madin Darby canine kidney (MDCK) cells by cytopathic effect reduction assay. They also showed antimicrobial activity against Gram-positive bacteria. This paper reports the taxonomy of the producing organism and the fermentation, isolation, physico-chemical properties and biological activities of kistamicins A and B. The structure determination will be described in the following paper[1].

Taxonomic Studies

Morphology

The substrate mycelium is well-branched and non-fragmenting (0.5 μm in diameter). Aerial mycelium is poorly formed on a limited number of media and bears monopodially branched chains of spores. The spore chain are short (10 to 20 spores per chain), mostly sessile with hooks or tightly closed spirals at the tip. Some tightly closed spirals are observed as pseudosporangia. The spores are spherical or oblong ($0.7 \sim 0.9 \times 0.8 \sim 1.5\,\mu$m), non-motile, and have a smooth surface.

Cultural and Physiological Characteristics[2,3]

The substrate mycelia is colorless, brownish pink to deep red. The aerial mycelium, if formed, is white. Melanoid and other distinct diffusible pigments are not formed. The temperature range for growth is 22°C to 45°C. Strain No. S328-8 grows on 3% but not 4% NaCl. It is sensitive to lysozyme. The cultural and physiological characteristics of strain No. S382-8 are shown in Tables 1 and 2, respectively.

Chemotaxonomy

Whole cell hydrolysate contains *meso*-diaminopimelic acid, ribose, madurose, mannose, galactose,

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Table 1. Cultural characteristics of strain No. S382-8[a].

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose - nitrate agar (Czapek-Dox agar)	Poor	Moderate; white	Colorless to brownish pink (33)[b]	None
Tryptone - yeast extract broth (ISP No. 1)	Moderate, not turbid	None	Colorless	None
Yeast extract - malt extract agar (ISP No. 2)	Good	None or scant; whitish	Very deep red (14)	None
Oatmeal agar (ISP No. 3)	Moderate	Scant; whitish	Grayish red (19)	Grayish pink (8)
Inorganic salts - starch agar (ISP No. 4)	Poor	None	Deep yellowish brown (75)	None
Glycerol - asparagine agar (ISP No. 5)	Poor	None	Brownish pink (33)	None
Peptone - yeast extract - iron agar (ISP No. 6)	Poor	None	Moderate reddish brown (43)	None
Tyrosine agar (ISP No. 7)	Poor	None or scant; white	Brownish pink (33)	None
Glucose - asparagine agar	Poor	None	Colorless	None
Nutrient agar	Poor	None	Very deep red (14)	None
BENNETT's agar	Moderate	None	Dark red (16)	None

[a] Observed after incubation at 28°C for 3 weeks.
[b] Color name and number in parentheses follow the color standards in ISCC-NBS Centroid Color Charts (United States Department of Commerce, National Bureau of Standards, 1985).

and glucose. Therefore, the cell wall belongs to Type III[4)]. Phospholipids present are phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside, and unknown glucosamine-containing phospholipids and, hence, belong to Type P-IV[5)]. The major menaquinone is MK-9 (H_4)[6)].

These chemotaxonomic and morphological characteristics indicate that strain No. S362-8 belongs in the genus *Microtetraspora*[7)]. Among the known species of this group, strain No. S382-8 shares many common characteristics with *M. parvosata*[8)]. However, as shown in Table 3, strain No. S382-8 is different from *M. parvosata* in some minor cultural and physiological characteristics. Thus, strain No. S382-8 is designated *Microtetraspora parvosata* subsp. *kistnae* subsp. nov. and was deposited in the American Type Culture Collection, Rockville, MD, under the accession number ATCC 55076.

Fermentation

A small chunk of a mature slant culture of *Microtetraspora parvosata* subsp. *kistanae* strain No. S382-8 (ATCC 55076) was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the seed medium [soluble starch (Nichiden Kagaku) 0.5%, glucose 0.5%, fish meat extract (Mikuni Kagaku) 0.1%, yeast extract (Oriental Yeast) 0.1%, NZ-case (Sheffield) 0.2%, NaCl 0.2%, and $CaCO_3$ 0.1%; pH was adjusted to 7.0 before sterilization]. This seed culture was incubated at 32°C for four days on a rotary shaker (200 rpm), and 5 ml portions of the culture was transferred into a 500-ml Erlenmeyer flask containing 100 ml of the production medium [glucose 2%, fish meal D30X (Banyu Eiyo) 1%, and $CaCO_3$; pH was adjusted to 7.0 before autoclaving]. The fermentation was carried out at 28°C for seven days on a rotary shaker (200 rpm). Antibiotic production in the fermentation broth was monitored by the conventional dye-uptake assay method[9)] using influenza virus type A. The fermentation broth showed the maximum

Table 2. Physiological characteristics of strain No. S382-8.

Hydrolysis of:		L-Rhamnose	+
Gelatin	+ (slow)	D-Glucose	+
Starch	Trace	D-Galactose	+
Milk coagulation	–	D-Fructose	+
Peptonization	–	D-Mannose	+
Production of:		L-Sorbose	–
Nitrate reductase	+	Sucrose	+
Tyrosinase	–	Lactose	+
Tolerance to:		Cellobiose	+
Lysozyme, 0.01%	–	Melibiose	+
NaCl, 3%	+	Trehalose	+
NaCl, 4%	–	Raffinose	+
pH, 5.3~10	+	D-Melezitose	–
Temperature:		Soluble starch	+
Growth range	22~45°C	Cellulose	–
Optimal growth	37~42°C	Dulcitol	–
No growth	19 and 48°C	Inositol	+[b]
Decomposition of:		D-Mannitol	+[b]
Adenine	–	D-Sorbitol	+
Casein	+	Salicin	+
Esculin	+	Acid from:	
Hippuric acid	+	Adonitol	+
Hypoxanthine	+	Arabinose	+
Tyrosine	+	Cellobiose	+
Urea	–	Erythritol	–
Xanthine	–	Glucose	+
Survival at 50°C, 8 hours:	–	Glycerol	+
Utilization of:		Inositol	+
Na-benzoate	–	Lactose	+
Na-citrate	–	Maltose	+
Na-mucate	–	Mannose	+
Na-succinate	+	Melezitose	–
Na-tartrate	–	Melibiose	+
Carbohydrate utilization of:[a]		Methyl α-D-glucoside	+[b]
Glycerol	+	Raffinose	+
D-Arabinose	+	Rhamnose	+
L-Arabinose	+	Sorbitol	–
D-Xylose	+	Trehalose	+
D-Ribose	+	Xylose	+

Abbreviation: +, positive characteristic; –, negative characteristic.
[a] Basal medium: PRIDHAM and GOTTLIEB's inorganic salts medium (ISP No. 9).
[b] Weakly positive.

antiviral activity, active at 48-fold dillution, after seven days. A scale-up fermentation was also carried out using stir-jar fermentors. A 500 ml portion of the seed prepared by the flask fermentation was transferred into a 20-liter stir-jar fermentor containing 12 liters of the production medium. The fermentation was carried out at 28°C for five days (113 hours) with stirring at 250 rpm and aeration rate of 12 liters per minute. The activity of the stir-jar fermentation was almost the same as that of the flask fermentation.

Extraction and Purification

The fermentation broth (49 liters) was stirred vigorously with 1-butanol (25 liters) for one hour. The organic layer was separated with a Sharpless centrifuge (Kokusan Seiko Co., No. 4A) and concentrated *in vacuo* to 300 ml. This was added dropwise to 1.5 liter of hexane and the precipitate formed was collected by centrifugation to give a crude solid (22.9 g). This solid was subjected to column chromatography on

Silica gel 60 (E. Merck, No. 9385, 40 i.d. × 750 mm) which was developed with ethyl acetate - methanol (1 : 1) and then with ethyl acetate - methanol - water (7 : 3 : 1). The eluate was collected in fractions and examined by TLC (ethyl acetate - methanol - water, 10 : 3 : 1, iodine detection). The main active fractions (kistamicin A, Rf 0.08) were combined, concentrated under reduced pressure and freeze-dried to yield a yellow powder (6.9 g). The minor active fractions (kistamicin B, Rf 0.27) were concentrated to give 4.1 g of crude kistamicin B.

One-half of the main powder was dissolved in a small volume of aqueous dimethyl sulfoxide and charged on a reversed phase silica gel column (YMC-GEL ODS-AM, Yamamura Chem. Lab. Co. Ltd., 40 i.d. × 500 mm) which had been equilibrated with acetonitrile - 0.2% KH_2PO_4 buffer pH 3.5 (3 : 17). Elution was carried out with the same mixture with a ratio of first 1 : 4 and then 1 : 3, and the eluate was monitored by HPLC (column: Cosmosil 5C18-AR, 4.6 i.d. × 250 mm, Nacalai Tesque; mobile phase: acetonitrile - 0.2% KH_2PO_4 buffer pH 3.5, gradient 0～20 minutes, 1 : 4～3 : 2; flow rate: 1 ml/minute; detection: UV 254 nm; Rt 11.8 minutes). The fractions containing pure kistamicin A were pooled, concentrated and extracted with 1-butanol. The extract was washed with water and evaporated *in vacuo* to give a pale yellow powder (2.2 g). The remaining crude powder was chromatographed in a similar manner (total 4.57 g). A portion of this powder (310 mg) was subjected to Sephadex LH-20 column chromatography (22 i.d. × 720 mm) eluting with methanol to afford a pure solid of kistamicin A (275 mg).

The crude kistamicin B was chromatographed on silica gel (40 i.d. × 750 mm) with elution of ethyl acetate - methanol - water (20 : 6 : 1). The active fractions were combined and evaporated (2.37 g). One forth of the solid (590 mg) was further purified on reversed phase silica gel (40 i.d. × 500 mm) eluting with acetonitrile - 0.2% KH_2PO_4 buffer pH 3.5 (2 : 3). Upon examination by HPLC (mobile phase:

Table 3. Differential characteristics between strain No. S382-8 and *Microtetraspora parvosata* SK & F-AAJ-271.

Test	Strain No. S382-8	*M. parvosata* SK & F-AAJ-271
Coproduction of purplish pigment in nutritionally rich organic media, *e.g.*, ISP media Nos. 2 and 3	−	+
Decomposition of:		
Adenine	−	+
Urea	−	+
Utilization of:		
Citrate	−	+
Acid from:		
D-Sorbitol	−	+

Abbreviation: +, positive characteristic; −, negative characteristic.

Table 4. Physico-chemical properties of kistamicins A and B.

	Kistamicin A	Kistamicin B
Nature	Pale yellow powder	Pale yellow powder
MP	>300°C (dec)	>300°C (dec)
$[\alpha]_D$	−1.8° (*c* 1.0, MeOH)	+22° (*c* 0.5, MeOH)
Molecular formula	$C_{61}H_{51}N_8O_{15}Cl$	$C_{70}H_{60}N_9O_{16}Cl$
HRFAB-MS [$(M+H)^+$, *m/z*]		
Calcd:	1,171.3240	1,318.3930
Found:	1,171.3290	1,318.3892
UV λ_{max} nm (ε)		
in MeOH	231 (sh, 53,700), 265 (sh, 20,700), 284 (14,900), 305 (sh, 11,200)	230 (sh, 52,000), 265 (sh, 20,000), 287 (16,400), 304 (sh, 12,700)
in 0.01 N NaOH - MeOH	244 (sh, 55,900), 286 (17,700)	243 (sh, 50,200), 291 (17,700)
IR ν_{max} (KBr) cm^{-1}	3400, 3300, 1650, 1510, 1400, 1225	3350, 1650, 1510, 1400, 1225

acetonitrile - 0.2% KH_2PO_4 buffer pH 3.5, gradient 0~20 minutes, 1:4~3:2; Rt 15.4 minutes), appropriate fractions were pooled, and then kistamicin B (220 mg) was recovered by 1-butanol extraction. After final purification by Sephadex LH-20 column chromatography, a homogeneous pale yellow powder of kistamicin B (164 mg) was obtained.

Physico-chemical Properties

Kistamicins A and B are soluble in dimethyl sulfoxide, methanol, and alkaline water, but practically insoluble in other solvents. They gave positive responses to iodine, sulfuric acid, ferric chloride, Rydon-Smith

Fig. 1. IR spectrum of kistamicin A (in KBr).

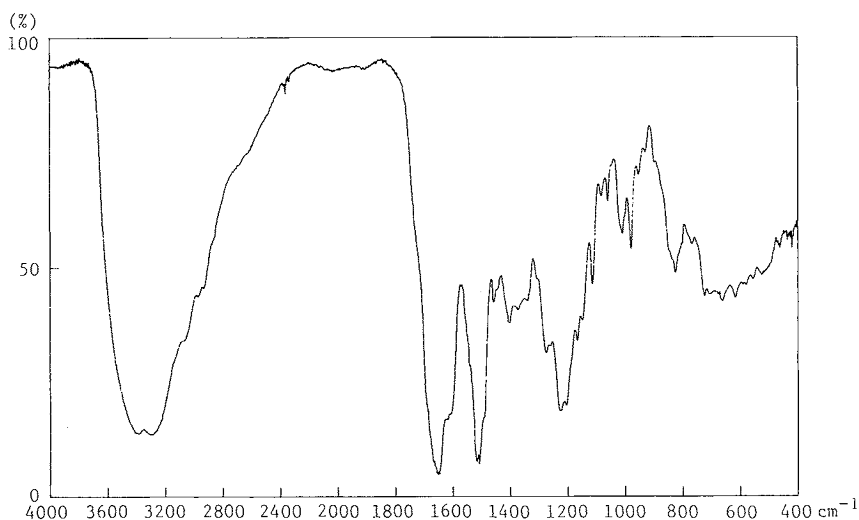

Fig. 2. ^1H NMR spectrum of kistamicin A (400 MHz, in DMSO-d_6).

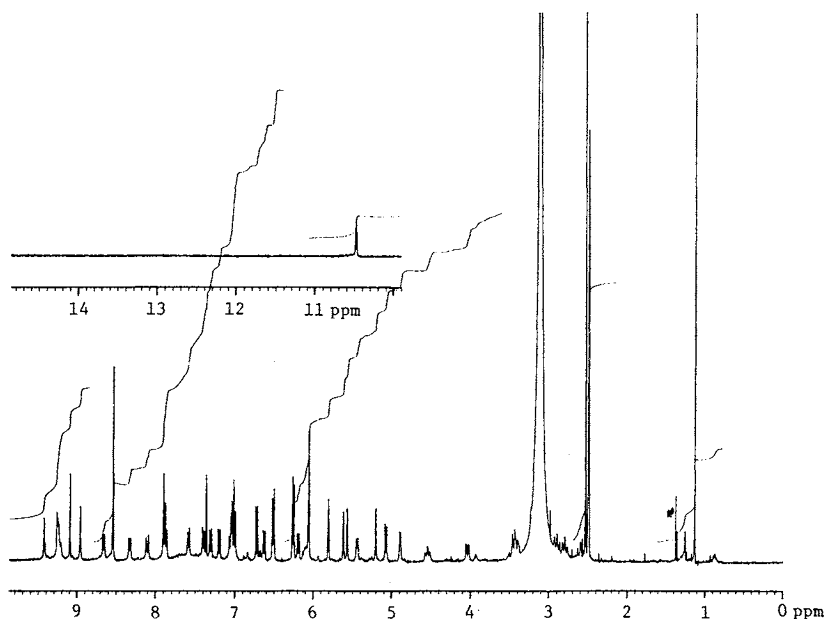

Fig. 3. ^{13}C NMR spectrum of kistamicin A (100 MHz, in DMSO-d_6).

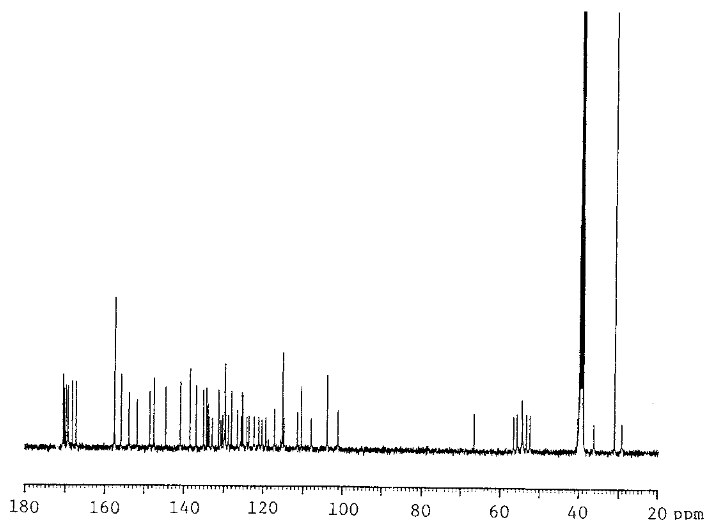

Table 5. Antiviral activities of kistamicins A and B.

	Influenza virus-MDCK cell		HSV-Vero cell		HIV-CEM-SS cell	
	ID_{50} (μg/ml)	TD_{50} (μg/ml)	ID_{50} (μg/ml)	TD_{50} (μg/ml)	ID_{50} (μg/ml)	TD_{50} (μg/ml)
Kistamicin A	3.6	>200	44	>200	>100	>100
Kistamicin B	1.8	>200	30	>200	>100	>100
Ribavirin	10	>100				
Acyclovir			0.25	>100		
2',3'-Dideoxyinosine (ddI)					60	>500

ID_{50}: Concentration to reduce CPE by 50% as compared to drug-free controls. TD_{50}: Concentration to inhibit the growth of host cells by 50%.

and Ehrlich tests but negative to anthrone test. The physico-chemical properties of both antibiotics are summarized in Table 4. The FAB-MS of kistamicin A gave the protonated molecular ion at *m/z* 1,171 $(M+H)^+$, whose isotope ion (*m/z* 1,173) pattern indicated the presence of one mole of chlorine atom. The molecular formulae of kistamicins A and B were assigned as $C_{61}H_{51}N_8O_{15}Cl$ and $C_{70}H_{60}N_9O_{16}Cl$, respectively by the HRFAB-MS. They showed similar UV absorption maxima in methanol, which exhibited bathochromic shift in alkaline solution. The IR, and ^{1}H and ^{13}C NMR spectra of kistamicin A are illustrated in Figs. 1, 2 and 3, respectively. The structures of kistamicins A and B have been determined by chemical and spectroscopic methods and are described in the following paper[1].

Biological Activity

In vitro antiviral activity of kistamicins A and B was assessed by the dye-uptake assay[9] using the influenza virus type A Victoria strain-Madin Darby canine kidney (MDCK) cell and herpes simplex virus (HSV) type 1 KOS strain-Vero cell systems[10]. Ribavirin and acyclovir were used as reference compounds in the anti-influenza virus and anti-HSV assay, respectively. The activity against human immunodeficiency virus (HIV) was determined by the XTT assay using CEM-SS cells infected with HIV (LAV_{BRU} strain

obtained from Luc Montagnier, Institute Pasteur, Paris, France) as described by WEISLOW *et al.*[11], 2′,3′-dideoxyinosine (ddI) was used as a positive control. The results are shown in Table 5. Kistamicins A and B demonstrated stronger anti-influenza virus activity than ribavirin. Kistamicin B exibited about two-fold greater antiviral activity than kistamicin A, indicating that the *N*-terminal substituent of the former enhanced the *in vitro* activity against influenza virus A. Both kistamicins A and B showed little or no antiviral activities against either HSV or HIV. They were also inactive in the syncytium formation inhibition assay in two cell lines: HeLa-T4 cells expressing CD4 antigen and BSC-1 cells expressing gp-120[12] (ID_{50} >100 μg/ml; dextran sulfate, ID_{50} 12.0 μg/ml). Kistamicins A and B exhibited very weak cytotoxicities against human colon carcinoma (HCT-116) and murine melanoma (B16-F10) cells with IC_{50} > 200 μg/ml.

Table 6. Antibacterial activity of kistamicin A.

Test organism	MIC (μg/ml)
Staphylococcus aureus 209P	12.5
S. aureus Smith	12.5
S. aureus D136	25
S. aureus No. 52-34	12.5
S. aureus A20239	25
S. aureus A9606	12.5
S. aureus A15097	12.5
S. aureus D153	6.3
S. aureus A22152	25
Enterococcus faecalis A9612	50
Micrococcus luteus PCI 1001	1.6
Bacillus subtilis PCI 219	6.3
Escherichia coli Juhl	>100

Antibacterial activity of kistamicin A was determined by the serial two-fold agar dilution method using nutrient agar medium (Eiken) and an inoculum of $10^3 \sim 10^4$ cfu/ml. It showed moderate activity against Gram-positive bacteria but no activity against Gram-negative bacteria (Table 6).

Discussion

Kistamicins A and B are novel antiviral antibiotics produced by *Microtetraspara parvosata* subsp. *kistanae* subsp. nov. that exhibited inhibitory activity against influenza virus type A and moderate antibacterial activity against Gram-positive bacteria *in vitro*. The structural studies[1] indicated that kistamicins are related to the nuclei of the vancomycin group antibiotics particularly to antibiotic complestatin. Complestatin was originally isolated as an anticomplement substance[13] and was later shown to inhibit HIV induced syncytium formation and cytopathicity[14]. Kistamicins A and B, however, did not inhibit either syncytium formation or the cytopathic effect caused by HIV in our assay. These findings clearly demonstrated the difference of the biological activities between kistamicin and complestatin in spite of their structural similarity. It is also of interest to note that vancomycin aglycone was inactive against influenza virus in our assay.

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