A NEW INHIBITOR OF PROTEIN KINASE C, RK-286C (4′-DEMETHYLAMINO-4′-HYDROXYSTAUROSPORINE)

I. SCREENING, TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITY

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(Received for publication September 12, 1989)

In the course of our screening program using a bleb-forming assay, a new inhibitor of protein kinase C (PKC) was found in the fermentation of a streptomycete. The inhibitor, RK-286C (4′-demethylamino-4′-hydroxystaurosporine), inhibited the morphological change of K562 cells, a human chronic erythroleukemia cell, induced by phorbol 12,13-dibutylate at the concentration of 3 μM. The same concentration of the compound inhibited the activity of PKC *in vitro* and the aggregation of rabbit platelets induced by collagen and arachidonic acid.

The phospholipid- and Ca^{2+}-dependent protein kinase C (PKC)[1] plays an important role as a regulatory element in signal transduction, cellular regulation, and tumor promotion[2,3]. The tumor-promoting phorbol esters and teleocidins are known to activate PKC directly[4,5]. Therefore, inhibitors of PKC are expected to be antitumor agents as well as tools for examining the mechanism of action of tumor-promotion and of PKC. In order to find inhibitors of PKC among microbial secondary metabolites, we developed a unique assay system, named the bleb-forming assay[6]. PKC activators[7] and inhibitors[8] could be detected by this assay system. In this report, we describe screening, taxonomy, fermentation of the producer actinomycete, and the biological activity of a new inhibitor, RK-286C (4′-demethylamino-4′-hydroxystaurosporine). Isolation and chemical characterization will be reported in the following paper[9].

Materials and Methods

Chemicals

RK-286C, staurosporine and K-252c were purified from the culture broth of strain RK-286. K-252a was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). Phorbol 12,13-dibutylate (PDBu), collagen, adenosine diphosphate (ADP) and arachidonic acid were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Screening

Cell culture of K562, a human leukemia cell, and bleb-forming assay were described in previous papers[6,8]. Ten μl of culture extracts of soil actinomycetes was added to 200 μl of K562 cell culture (1×10^5 cells/ml) with 0.2 μg of PDBu.

Fifteen minutes after addition of PDBu, the morphology of K562 cells was observed under a microscope. If the fermentation broth contained PKC inhibitors, the morphological change of K562 cell surface induced by PDBu was inhibited.

HPLC conditions for the separation of indolocarbazole group antibiotics was as follows; column: Capcell Pak (4.6 i.d. $\times$ 250 mm, Shiseido, Tokyo), solvent system: Methanol - water - 1% NH_4OH (8 : 1 : 1),

flow rate: 1 ml/minute, detection: UV 292 nm.

Taxonomy

Taxonomic characterization of strain RK-286 was done using the methods and media of International Streptomyces Project (ISP)[10]. The color of mycelia was identified by using the Color Harmony Manual (4th Ed., 1958, Container Corporation of America, Chicago, Illinois). Whole-cell sugars were identified by the method of LECHEVALIER and LECHEVALIER[11] and diaminopimelic acid isomers were analyzed by the method of BECKER *et al.*[12].

Fermentation

Strain RK-286 was cultured in 500 ml cylindrical flasks containing 70 ml of fermentation medium (glucose 0.5%, soybean meal 2.5%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4% and NaCl 0.2%, adjusted to pH 7.0). The fermentation was carried out on a rotary shaker (250 rpm) at 28°C for 5 days.

Antimicrobial Activity of RK-286C

Potato - sucrose agar medium was used for the culture of fungi and *Xanthomonas campestris*. Bacteria were cultured in nutrient agar medium. MICs were determined by the conventional agar dilution method.

Biological Activity of RK-286C

Inhibitory activity of various members of indolocarbazole antibiotics against PKC *in vitro* was analyzed by the method described in previous papers[6,8]. Quantitative analysis was carried out by measuring the density of bands in autoradiography.

Inhibition assay of platelet aggregation by RK-286C and related compounds was carried out by the procedure of UMEHARA *et al.*[13]. Platelets were prepared from a New Zealand white rabbit (2.5 kg, male). It was observed under a microscope that collagen (50 μg/ml), ADP (5 μM) and arachidonic acid (100 μg/ml) induced 80 to 90% of platelets aggregation.

Results and Discussion

Screening

We found that bleb suppressing compounds were produced by several strains. Most of these compounds were identified as staurosporine[14] which shows an extremely low inhibition dose (ID_{50} 3 nM) on the bleb formation as well as on PKC activity *in vitro*.

An actinomycete isolated in Nanao-shi, Ishikawa Prefecture, Japan was found to produce bleb suppressing compounds. These compounds were analyzed by HPLC and revealed that the compounds included staurosporine[14], K-252c[15] and a new member of the indolocarbazole group of antibiotics, RK-286C (4'-demethylamino-4'-hydroxystaurosporine)[9]. The retention time on the HPLC analysis of these compounds was 8.14, 5.0, and 6.23 minutes, respectively.

Taxonomy of the Producing Strain

The producing strain, RK-286, was cultured on various ISP media and the characteristics are summarized in Table 1. Strain RK-286 utilized D-glucose, L-rhamnose, raffinose, sucrose, inositol and D-mannitol. The strain did not utilize L-arabinose, D-fructose and D-xylose.

Strain RK-286 has spiral spore chains of the Retinaculum-Apertum type (Fig. 1). The spore surface ornamentation is smooth and its shape is cylindrical, averaging 1.3 × 0.6 μm in size.

The whole-cell sugar composition of strain RK-286 was found to be galactose, glucose and ribose, which sugar pattern is the Type NC[16]. The cell hydrolysate contained the L,L-isomer of diaminopimelic acid, which is cell-wall type I[11].

Based on these chemotaxonomic and general characteristics, the strain is considered to belong to the

Table 1. Cultural characteristics of strain RK-286.

Medium	Growth	Reverse color	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP No. 2)	Good	Mustard gold (2ne)	Dusk (10fe)	None
Oatmeal agar (ISP No. 3)	Good	Orchard haze (10dc)	Dusk (10fe)	None
Inorganic salts-starch agar (ISP No. 4)	Good	Lt wheat (2ea) + natural string (3dc)	Pearl (3ba) + dawn pink (7dc)	None
Glycerol-asparagine agar (ISP No. 5)	Good	Ivory tint (2cb)	Pale yellow (1ca)	None
Peptone-yeast extract-iron agar (ISP No. 6)	Moderate	Deep brown (4pl)	Deep brown (4pl)	Deep brown (4pl)
Tyrosine agar (ISP No. 7)	Moderate	Black plum (10po)	Light gray (c)	Deep brown (4pl)
Starch-yeast extract agar	Good	Dull gold (2ng)	Dusk (10fe)	None
Nutrient agar	Good	Bamboo (2gc)	Bamboo (2gc)	None
Glucose-asparagine agar	Moderate	Ivory tint (2cb)	Dusk (10fe)	None
Sucrose-nitrate agar	Poor	White (a)	White (a)	None

Fig. 1. Scanning electron microscopy of strain RK-286.

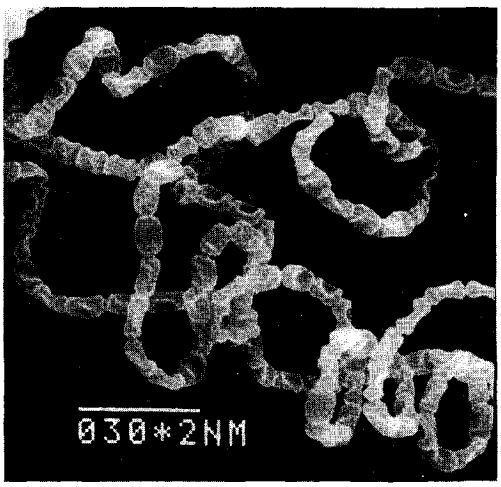

Table 2. Effect of carbon source on the production of RK-286C.

		Production titer (μg/ml)		
		RK-286C	Staurosporine	K-252c
Control	(without sugar)	5.8	18.3	1.7
Glucose	0.5%	9.2	20.0	0.5
Glucose	1.0%	7.8	29.0	0.8
Glucose	2.0%	6.2	35.0	1.3
Glycerol	2.0%	—	25.0	—
Fructose	2.0%	—	5.8	2.1
Maltose	2.0%	7.5	26.7	1.7
Lactose	2.0%	4.2	58.3	1.1
Sucrose	2.0%	6.7	50.0	0.8

The basal medium for this assay consisted of soybean meal 2.5%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4%, NaCl 0.2% and the sugar listed.

genus *Streptomyces*. The strain was similar to *Streptomyces resistomycificus*, *Streptomyces massasporeus*, *Streptomyces aurantiogriseus* and *Streptomyces griseosporeus* which are described in BERGEY's Manual[17]. However, direct comparison was not carried out.

Fermentation

Streptomyces sp. RK-286 produced several indolocarbazole antibiotics which differ in their sugar moiety. When we attempted to increase the production of RK-286C, it was revealed that the production titer of the antibiotic was influenced by the carbon source in the fermentation medium. When glucose was replaced with fructose, the production of both RK-286C and staurosporine was drastically reduced (Table 2). Among these conditions, the best titer of RK-286C was obtained when the strain was cultured in the medium containing 0.5% of glucose.

Time course of the fermentation was monitored in various conditions. When the growth of the strain

Table 3. Antimicrobial spectrum of RK-286C.

Organisms tested	MIC (μg/ml)	Organisms tested	MIC (μg/ml)
Pyricularia oryzae IFO 5994	100	*Escherichia coli* AB 1157	>200
Botryotinia fuckeliana IFO 5365	>200	*E. coli* BE 1186	>200
Alternaria mali IFO 8984	>200	*Salmonella typhimurium* SL1102	>200
Colletotrichum lagenarium	>200	*Pseudomonas aeruginosa* IFO 13130	>200
Candida albicans	>200	*Staphylococcus aureus* IFO 12732	>200
Xanthomonas campestris pv. *citri*	>200		

Fungi and *X. campestris* were cultured on potato-sucrose agar at 27°C for 7 days. Bacteria were cultured on nutrient agar supplemented with yeast extract 0.2% at 37°C for 3 days.

Table 4. Inhibitory activities of indolocarbazole antibiotics.

	IC_{50} (μM)	
	Bleb formation[a]	PKC[a]
RK-286C	3.3	3.0
Staurosporine	0.003	0.003
K-252a	0.01	0.01
K-252c	3.0	3.0

[a] K562 cells and PKC were activated with 1.0 and 10 μg/ml of PDBu, respectively.

Table 5. Inhibitory activities of indolocarbazole antibiotics.

	IC_{100} of platelet-aggregation (μM)		
	Collagen	ADP	Arachidonic acid
RK-286C	5	30	5
Staurosporine	30	10	5
K-252a	50	50	>100
K-252c	>100	>100	>100

The concentrations of platelet-aggregation inducers were as follows; collagen, 50 μg/ml; ADP, 5 μM; arachidonic acid, 100 μM.

was rapid, production of staurosporine increased. In contrast, when the growth rate of the producer was restricted by limited carbon source or aeration, production of RK-286C was enhanced.

Biological Activity

Antimicrobial activity of RK-286C was very weak; the growth of *Pyricularia oryzae* IFO 5994 was inhibited at a concentration of 100 μg/ml (Table 3). Antibacterial activity of the antibiotic was not observed among the strains tested. It was reported that staurosporine showed strong antifungal and weak antibacterial activity and that K-252a and K-252c had no antimicrobial activity.

Inhibitory activity of RK-286C against PKC as well as bleb formation induced by PDBu was compared with other related compounds (Table 4). Staurosporine, K-252a, K-252c and RK-286C have the same chromophore. Alteration was only in the sugar moiety. Staurosporine showed the strongest inhibition in both assays (PKC inhibition and bleb suppression of K562 cells induced by PDBu). These data suggested that the sugar moiety of indolocarbazole group antibiotics is important for biological activity.

The inhibitory activity of RK-286C against PKC as well as bleb formation was three orders of magnitude weaker than that of staurosporine, and comparable to those of H7[6] and sangivamycin[8].

It was reported that staurosporine[13] and K-252a[18] inhibited the aggregation of platelets. Inhibitory activity of platelet-aggregation of RK-286C and other related compounds was tested by using collagen, ADP and arachidonic acid as aggregation inducers. As described in Table 4, the inhibition activity of RK-286C against PKC was much weaker than that of staurosporine. However, it is worth noting that the inhibition of platelet-aggregation of RK-286C was as strong as that of staurosporine (Table 5). These observations suggested that the inhibitory action against platelet-aggregation is independent of the inhibition of PKC activity. RK-286C may be regarded as a potential anti-inflammatory substance and also a useful

tool to analyze the physiological function of PKC *in vivo*.

Acknowledgments

We are grateful to Dr. T. MIZUTANI and Ms. S. MIURA (RIKEN) for their help to prepare the platelet sample from rabbits. We thank Drs. Y. MATSUDA and K. YAMADA (Kyowa Hakko Kogyo Co., Ltd.) for their advice to measure the inhibition of platelet-aggregation. This work was carried out as a part of the project research for Life Science promoted by The Institute of Physical and Chemical Research (RIKEN) and a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

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