A NEW INHIBITOR OF PROTEIN KINASE C, RK-1409 (7-OXOSTAUROSPORINE)

I. TAXONOMY AND BIOLOGICAL ACTIVITY

HIROYUKI OSADA, HIROYUKI KOSHINO, TAKUJI KUDO, RIE ONOSE and KIYOSHI ISONO*

RIKEN, The Institute of Physical and Chemical Research, Wako, Saitama 351-01, Japan

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A novel inhibitor of protein kinase C was found in the fermentation of a soil actinomycete, strain RK-1409. According to the taxonomic studies, the producing strain was designated as *Streptomyces platensis* subsp. *malvinus* RK-1409. The protein kinase C inhibitor, RK-1409 (7-oxostaurosporine) inhibited the morphological change of a human chronic erythroleukemia cell, K-562, induced by phorbol 12,13-dibutyrate (PDBu) at the concentration of 10 ng/ml. The concentration of 3 ng/ml inhibited the activity of protein kinase C *in vitro*. RK-1409 inhibited the cell cycle progression at G_2 phase of K-562 cells.

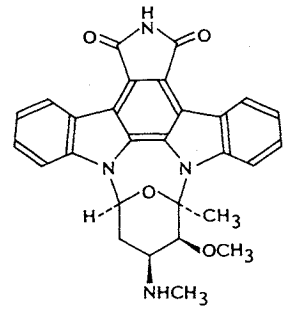
Protein kinase C which requires phospholipid and Ca^{2+} for its activity plays important roles as a regulatory element in signal transduction, cellular regulation, and tumor promotion[1,2]. The tumor-promoting phorbol esters and teleocidins directly activate protein kinase C[3,4]. We have established a unique screening system termed as the bleb forming assay[5] to detect protein kinase C inhibitors based on the morphological change of a human chronic leukemia cell line, K-562. We have reported that protein kinase C inhibitors, sangivamycin[6], RK-286C[7], and RK-286D[8] were detected by this assay system. In this report, we describe taxonomy of the producer strain and biological activity of a new compound, RK-1409 (Fig. 1). Isolation, physico-chemical properties and chemical structure will be presented in the following paper[9].

Materials and Methods

Taxonomic Studies

Methods and media recommended by International Streptomyces Project (ISP)[10] were used to examine the taxonomic characterization of strain RK-1409. Morphology on ISP media was observed after incubation at 28°C for 14 days. The Color Harmony Manual (4th Ed., 1958, Container Corporation of America, Chicago, Illinois) was used to identify the color of mycelial and soluble pigments. A scanning electron microscope (SEM) was used to study morphology of the spore chains. 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].

Fig. 1. Structure of RK-1409 (7-oxostaurosporine).



Bacterial Strain

Strain RK-1409 has been deposited at the Fermentation Research Institute, Agency of In-

Table 1. Cultural characteristics.

		RK-1409	JCM 4189	JCM 4914	JCM 4953
Yeast extract - malt extract agar (ISP No. 2)	G:	Abundant	Good	Good	Good
	RC:	Gold (1½nc)	Gold (2lc)	Gold (1½nc)	Gold (2lc)
	AM:	Good	Good	Good	Good
		White (a)	Pearl (3ba)	White (a)	Pearl (3ba)
	SP:	None	None	None	None
Oatmeal agar (ISP No. 3)	G:	Moderate	Good	Good	Good
	RC:	Pearl (3ba)	Light apricot (4ea)	Mustard tan (2lg)	Light wheat (2ea)
	AM:	Good	Good	Good	Good
		Natural (3dc)	Orchard tan (10ba)	Covert tan (2ge)	Mustard brown (2pl)
	SP:	None	None	None	None
Inorganic salts - starch agar (ISP No. 4)	G:	Good	Good	Good	Good
	RC:	Pearl (3ba)	Pearl (3ba)	Pearl (3ba)	Pearl (3ba)
	AM:	Good	Poor	Good	Poor
		White/Charcoal gray (a/w)	White (a)	White/Charcoal gray (a/w)	White (a)
	SP:	None	None	None	None
Glycerol - asparagine agar (ISP No. 5)	G:	Good	Poor	Good	Moderate
	RC:	Deep brown (5pl)	Yellow tint (1ba)	Deep brown (5pl)	Shell (3ca)
	AM:	Moderate	None	Poor	None
		White (a)		Oyster gray (b)	
	SP:	Brick red (5ng)	None	Cococa brown (5lg)	None
Peptone - yeast extract - iron agar (ISP No. 6)	G:	Good	Good	Good	Good
	RC:	Light wheat (2ea)	Light wheat (2ea)	Light wheat (2ea)	Light wheat (2ea)
	AM:	Moderate	None	Moderate	Poor
		Sand (3cb)		Shell pink (5ba)	Sand (3cb)
	SP:	None	None	None	None
Tyrosine agar (ISP No. 7)	G:	Good	Good	Abundant	Abundant
	RC:	Shell (3ca)	Shell (3ca)	Maize (2ia)	Maize (2ia)
	AM:	Good	Good	Abundant	Abundant
		White (a)	White (a)	White (a)	White (a)
	SP:	None	None	None	None
Glucose - asparagine agar	G:	Good	Good	Good	Good
	RC:	Redwood (6ie)	Pearl (3ba)	Redwood (6ie)	Pearl (3ba)
	AM:	Good	Good	Abundant	Good
		White/Gray (a/g)	Pearl (3ba)	Pearl (2ba)	Ivory tint (2cb)
	SP:	Pale lilac (11ca)	None	Redwood (6ie)	None
Sucrose - nitrate agar	G:	Moderate	Good	Good	Good
	RC:	Mustard gold (2ne)	Shell pink (5ba)	Mustard gold (2ne)	Light yellow (1ea)
	AM:	Good	Good	Good	Good
		White/Natural (a/3dc)	White (a)	White/Natural (a/3dc)	White (a)
	SP:	None	None	None	None
Starch - yeast extract agar	G:	Good	Good	Good	Good
	RC:	Mustard gold (2ne)	Light melon yellow (1ba)	Light antique gold (1½ic)	Gold (1½lc)
	AM:	Good	Good	Good	Good
		White/Gray (a/g)	Ashes (5fe)	Oyster white/Gray (b/i)	Light gray/Coffee (c/3pn)
	SP:	None	None	None	Maize (2ia)
Nutrient agar	G:	Good	Good	Good	Good
	RC:	Gold (1½nc)	Gold (1½lc)	Light wheat (2ea)	Maize (2ga)
	AM:	Moderate	None	None	Poor
		White (a)			Oyster gray (b)
	SP:	None	None	None	None

dustrial Science and Technology, Japan, under the accession number FERM-P11952. *Streptomyces platensis* JCM 4189, *S. platensis* subsp. *malvinus* JCM 4914, and *S. platensis* subsp. *clarensis* JCM 4953 were obtained from Japan Collection of Microbiology (JCM).

Bleb Forming Assay and Inhibition of Protein Kinase C

The bleb forming assay utilizing K-562 human leukemia cells was described in previous papers[5,6]. Ten microliters of the sample solution was added to 200 μl of cell culture (1×10^5 cells/ml) with 0.2 μg of phorbol 12,13-dibutyrate (PDBu). Fifteen minutes after addition of PDBu, morphology of K-562 cells was observed under a microscope.

Protein kinase C activity *in vitro* was assayed by the protein kinase C assay kit (Amersham). Assay conditions followed the procedure recommended by the supplier. The assay kit contains protein kinase C from bovine brain.

Flow Cytometer

Distribution of DNA contents depending the cell cycle was analyzed by a flow cytometer (Epics Profile II, Coulter). DNA in an isolated nuclei was stained with propidium iodide (Sigma).

Antimicrobial Activity

Antimicrobial activity was measured by the conventional paper disk agar plate method. Potato-sucrose agar and nutrient agar were used for measurement of antifungal activity and antibacterial activity, respectively.

Results and Discussion

Taxonomic Studies

The producing strain RK-1409 (FERM-P11952) was isolated from a soil sample collected in Atou, Yamaguchi Prefecture, Japan. The strain was cultured on various ISP media and the cultural characteristics are summarized in Table 1. Spotted gray colonies in white colonies were observed on some media. Soluble pigments were produced on asparagine containing media (ISP No. 5 and glucose-asparagine agar). Strain RK-1409 had spiral spore chains. The spore was characterized by its crescent shape as shown in Fig. 2 (average 0.8×0.56 μm in size), and its surface was smooth.

The whole cell hydrolysate contained the L,L isomer of diaminopimelic acid which corresponds to cell-wall type I[11]. The whole cell sugar composition of strain RK-1409 was found to be galactose, glucose and ribose, which is Type NC[13] sugar pattern.

According to BERGEY's Manual[14] and the ISP description, these chemotaxonomic and general characteristics of strain RK-1409 resemble those of *Streptomyces platensis*. Therefore, the cultural characteristics of strain RK-1409 were compared side by side with those of *Streptomyces platensis* JCM 4189, *S. platensis* subsp. *malvinus* JCM 4914, and *S. platensis* subsp. *clarensis* JCM 4953, (Tables 1 and 2).

Strain RK-1409 and *S. platensis* subsp. *malvinus* JCM 4914 formed the characteristic spotted colonies and soluble pigments on the asparagine containing media. Both strains utilized most carbon sources tested. In these respects, strain RK-1409 is most similar to *S. platensis* subsp. *malvinus* JCM 4914 among the tested strains, therefore, RK-1409 was designated as *S. platensis* subsp. *malvinus* RK-1409.

Inhibition of Protein Kinase C Activity *In Situ* and *In Vitro*

Inhibitory activity of RK-1409 against protein kinase C as well as bleb formation induced by PDBu was compared with that of staurosporine. Staurosporine is known to be the strongest inhibitor among

Fig. 2. Scanning electron micrograph of spore chains of strain RK-1409.

Bars in pictures indicate 3 μm (A) and 1.3 μm (B), respectively.

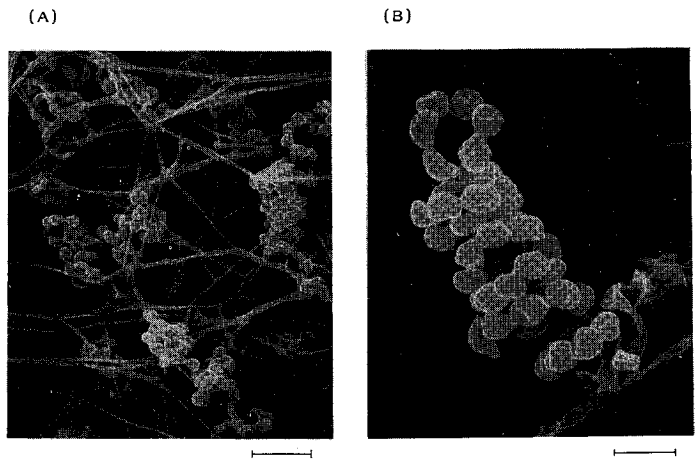


Table 2. Utilization of carbon source.

	RK-1409	JCM 4189	JCM 4914	JCM 4953
L-Arabinose	−	−	+	+
D-Fructose	+ +	+ +	+ +	+ +
D-Galactose	+	+ +	+ +	+ +
D-Glucose	+	+ +	+	+ +
Inositol	+ +	+ +	+ +	+
D-Mannose	+ +	+ +	+ +	+ +
Raffinose	+ +	+ +	+ +	+ +
L-Rhamnose	+	−	+	−
Sucrose	+ +	+ +	+ +	+
D-Xylose	+	−	+	−

Fig. 3. Inhibition of morphological change (bleb formation) of K-562 cells induced with PDBu.

Inhibitors, RK-1409 (○) and staurosporine (●), were added simultaneously with PDBu.

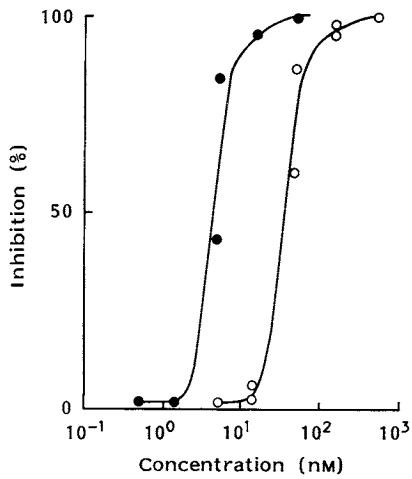
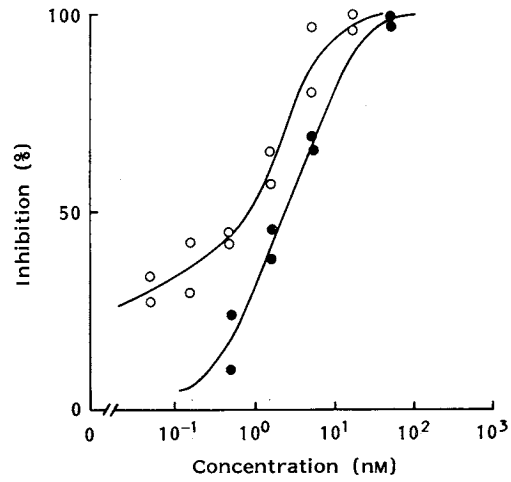


Fig. 4. Inhibition of protein kinase C activity *in vitro* by RK-1409 and staurosporine.

○ RK-1409, ● staurosporine.



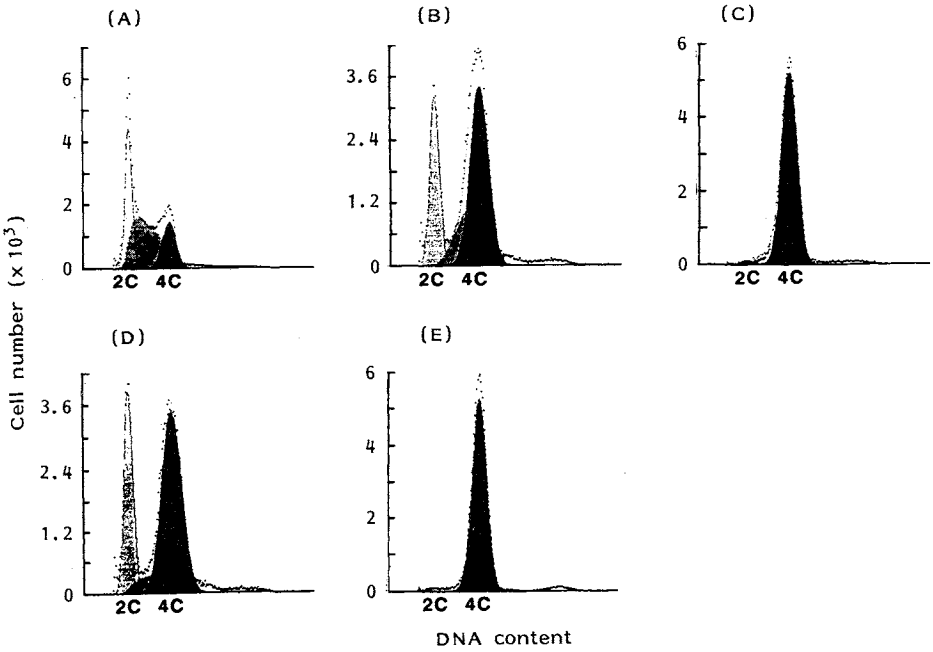
the microbial secondary metabolites[15]. As shown in Fig. 3, RK-1409 inhibited the bleb formation at 30 ng/ml, which is 3 ~ 10-fold higher concentration than that of staurosporine. The inhibition of protein kinase C *in vitro* by RK-1409 was more potent than staurosporine (Fig. 4).

Inhibition of the Cell Cycle Progression

It was reported[16,17] that indolocarbazole derivatives, staurosporine, RK-286C, and K-252a, inhibited the cell cycle progression. Staurosporine

Fig. 5. Inhibition of the cell cycle progression from G_2/M to G_1 phase.

K-562 cells were treated with RK-1409 and staurosporine at the following concentrations: (A) control, (B) RK-1409 30 ng/ml, (C) RK-1409 300 ng/ml, (D) staurosporine 10 ng/ml, and (E) staurosporine 100 ng/ml.



After 24 hours incubation, DNA content was measured by a flow cytometer. Cells in G_1/G_0 phase contain 2C DNA and cells in G_2/M phase contain 4C DNA. Cells in S phase contain DNA between 2C and 4C.

accumulated the G_2 cells with a 4C DNA content (diploid)[16]. In contrast, RK-286C and K-252a accumulated the G_2 cells with a polyploid[17]. We have tested if RK-1409 inhibits the cell cycle progression. As shown in Fig. 5, RK-1409 caused the G_2 arrest (minimal effective dose: MED 30 ng/ml) with 4C DNA cells. MED of RK-1409 was 3-fold higher than that of staurosporine and the polyploid cells were not observed.

In vitro inhibition of protein kinase C by RK-1409 was caused at the lower concentration compared with staurosporine, however, *in situ* (bleb formation and cell cycle progression) inhibition by RK-1409 was weaker than staurosporine. These results suggested that the membrane permeability of RK-1409 was lower than that of staurosporine.

Table 3. Antimicrobial spectrum of RK-1409.

Organisms tested	Inhibition diameter (mm)	
	RK-1409	Staurosporine
Chlorella vulgaris	19	27
Pyricularia oryzae IFO 5994	13	15
Botryotinia fuckeliana IFO 5365	—	11
Alternaria mali IFO 8984	—	—
Xanthomonas campestris pv. *citri*	—	—
Escherichia coli AB1157	—	—
E. coli BE1186	—	—
Salmonella typhimurium TV119	—	—
Pseudomonas aeruginosa IFO 13130	—	—
Staphylococcus aureus IFO 12732	—	—
Mycobacterium phlei IFO 3158	—	—

Antimicrobial activity of RK-1409 was weaker than that of staurosporine, and the growth of *Chlorella vulgaris* and *Pyricularia oryzae* was inhibited at the concentration of 40 μg/disk.

Acknowledgments

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