Fascinating Bioactive Compounds from Actinomycetes

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INTRODUCTION

Antibiotics were originally known as antimicrobial compounds which were produced by microorganisms. Nowadays, the definition of antibiotics has broadened to include antitumor, herbicidal and antiviral compounds. Moreover, enzyme inhibitors and receptor antagonists are

also categorized into the new concept of antibiotics.

Microorganisms can produce anything we want, if we know how to ask them. Therefore, "screening systems", namely, the methods to detect desirable biological activities are of crucial importance in search for bioactive compounds from microbial metabolites. A number of methods for detection of biological and pharmacological activities have already appeared¹⁻³⁾. In this paper, I will describe one of the characteristic examples of screening methods in our laboratory.

The success of antiviral chemotherapy has been limited to a few viral diseases. This limited success is due to several different reasons which underlie the difficulties in the development of effective antiviral agents. *In vitro* antiviral screening systems are usually primary step to find a new lead compounds with significant activity. Primary screening *in vitro* offers several advantages over *in vivo* methodologies in that large numbers of compounds may be screened quickly and economically.

SCREENING

We have established a convenient screening method utilizing the bacteriophage B and *Streptomyces griseus*. The basic idea of the bioassay was derived from the conventional paper disk agar plate method was used for the assay as shown in Fig. 1. The double layered agar plate containing *S. griseus* infected with phage B⁴⁾. When the test sample contains the inhibitor of

Fig. 1. The procedure of antibacteriophage assay based on the conventional agar plate - paper disk method.

bacteriophage replication, the survival ring of *S. griseus* around the paper disk was observed (Fig. 2). Daunomycin which is an antileukemic antibiotic and a DNA intercalater has strong antibacterial activity as well as antiphage activity. Therefore, two rings around the paper disk were observed; the inner cleared ring is an antimicrobial ring and the outer white ring is the survival ring of streptomycetes (Fig. 2B). During the screening, we discovered two novel antibiotics, RK-1441 and enopeptin. They had weak or no antibacterial activity but showed strong antiphage activity (Fig. 2A).

RK-1441

The isolation procedure of RK-1441 A and B⁵⁾ was summarized in Fig. 3. From 3-liter fermentation broth, 12 mg of A and 43 mg of B were purified by the successive column chromatographies and HPLC. Physico-chemical properties of RK-1441 A and B resembled each other (Table 1). From the UV absorption spectra, it was expected that the chromophore of RK-1441 A and B seems to be a benzodiaze-

pine derivative. Chemical structures of RK-1441 A and B were determined by the spectroscopic data, especially NMR data. RK-1441 A has a similar structure to neothramycin⁶⁾ except methoxy group at C-3 position (Fig. 4). Neothramycin was reported as a dehydrated form at C-11 position. RK-1441 A was purified as a carbinol amine form. RK-1441 B takes an amide form. As described later, this moiety in the structure plays a very important role in the biological activity.

Antiphage activities of RK-1441 A and B against two kinds of host-phage systems: one is *S. griseus* and phage B, the other one is *Escherichia coli* and phage T4 were compared with

Fig. 2. The typical profiles of antibacteriophage activity observed in the bioassay. Following inhibitors were identified or purified by the bioassay. A: RK-1441, enopeptin, neothramycin, B: daunomycin, chartreucin, mithramycin, novobiocin, C: rifamycin, destomycin, erythromycin, bafiromycin, D: staurosporine, herbimycin, E: tautomycin, factumycin, F: control (without inhibitor).

Fig. 3. The isolation procedure of RK-1441 A and B.

Table 1. Physico-chemical properties of RK-1441 A and B.

	RK-1441A	RK-1441-B
Appearance :	Pale yellow powder	Pale yellow powder
MP (°C) :	115~120 (dec)	224~235
$[\alpha]_D^{21}$:	+ 692° (c 0.52, MeOH)	+ 219° (c 0.54, EtOH)
HR-EI-MS (<i>m/z</i>) :	276.1101 ($M^+ - H_2O$)	278.0903 (H^+)
MF :	$C_{14}H_{18}N_2O_5$	$C_{13}H_{14}N_2O_5$
MW :	294	278
UV $\lambda_{max}^{50\% EtOH}$ nm(e):	235 (31,000) 264 (11,000) 318 (5,900)	235 (55,000) 267 (sh 15,400) 300 (8,600) 310 (sh 6,600)

Neothramycin

RK-1441A

RK-1441B

Fig. 4. Structures of neothramycin, RK-1441 A and B.

neothramycin. All the inhibitors inhibited the replication of both bacteriophages and RK-1441 A is more potent than RK-1441 B in the anti-phage activity (Table 2).

As neothramycin is known to be an antitumor and antiviral antibiotic, the antiviral effect of RK-1441A and B were examined. NIH/3T3 cells were infected with murine sarcoma virus which carries activated oncogene *H-ras*. After 2 weeks infection, the cell morphology was observed under a microscope. Control cells without inhibitor were transformed with retrovirus and showed the malignant morphology. On the other hand, the cells treated with inhibitors showed the flat normal morphology.

Next, we investigated the mechanism of action of RK-1441. Already it was reported that anthramycin group antibiotics covalently bind to DNA strand⁷⁾. Then we tested whether RK-1441 A and B bind to DNA or not. If the inhibitor covalently binds to DNA strand, restriction enzyme cannot cut the DNA. After incubation of DNA with inhibitors, the DNA was digested by various kind of restriction enzymes. When

SspI which recognition sequence does not contain guanine residues was used, DNA was completely digested by the enzyme. On the other hand, when we used the restriction enzyme, *Hind*III which recognition sequence contains guanine residues, the DNA treated with neothramycin and RK-1441 A was not digested by the enzyme (Fig. 5). This data suggest that the inhibitors bind guanine residues in DNA strands. RK-1441 B has no effect in this assay.

From the data above mentioned, the schematic model of the mode of action of RK-1441 A and B was illustrated (Fig. 6). RK-1441 A can directly bind to DNA *in vitro* and inhibited phage or viral replication in host cells. On the other hand, RK-1441 B could not bind DNA *in vitro*, however, the inhibitor showed antiviral and antiphage activity *in vivo*. To explain this contradictory phenomena, we must suppose RK-1441 B to be converted to the active form in the host organisms. These two compounds RK-1441A and B are very useful to study the mechanism of antiphage activity of benzodiazepine antibiotics.

Table 2. Inhibition of bacteriophage growth by RK-1441.

System	Minimal inhibition dose ^a			
	A		B	
	Phage ^b	Host ^c	Phage	Host
RK-1441A	25	>400	50	>400
RK-1441B	50	>400	100	>500
Neothramycin	6.3	50	12.5	200

System A : phage B / *Streptomyces griseus*. System B : T4 phage / *E. coli* AB1157. a: In the assay systems, the modified agar-plate/paper-disk method was used to measure the minimal inhibition dose (μg / disk) for bacteria and bacteriophages. b: Minimal inhibition dose against the bacteriophage growth. c: Minimal inhibition dose against host bacteria.

Fig. 5. The effect of RK-1441 to the activity of restriction enzymes.

Enopeptin

New depsipeptide antibiotics, enopeptins A and B were isolated as antiphage antibiotics from the culture broth of *Streptomyces* sp. RK-1051^{8,9)}. The isolation procedure of enopeptins A and B was summarized in Fig. 7. From 18-liter fermentation broth, 25 mg of enopeptin A and 10 mg of enopeptin B were purified.

Physico-chemical properties of enopeptins A and B were almost identical as shown in Table 3. From the extensive analyses of NMR and mass spectrometry, the structures of enopeptins A and B were determined as shown in Fig. 8.

Enopeptin A has a cyclic depsipeptide moiety which consists of 5 amino acids; serine, proline, N-methylalanine, alanine, and methylproline. Enopeptin B has a proline instead of methylproline in the cyclic depsipeptide moiety. This structural difference between enopeptin A and B is no influence on the biological activity.

Antiphage activity of enopeptins A and B against two host-phage systems were examined. Enopeptins A and B had strong activity against phage B replication in *Streptomyces* system, however the antibiotics had no effect on T4 phage in *E. coli* system (Table 4). As the

molecular size of enopeptins is larger than RK-1441 A, the membrane permeability of enopeptins may be poor in *E. coli*. The mode of action of enopeptins is different from that of RK-1441 A because enopeptin didn't bind to DNA.

Next the antiviral activity of enopeptin A on the tobacco mosaic virus was investigated. Tobacco mosaic viruses were inoculated on the leaf of *Nicotiana*, which was cut into two pieces. One of them was dipped into control solution without inhibitor, the other one was dipped into enopeptin solution. After 1 day and 2 days, the number of spots which was caused by viral infection was counted. At 500 $\mu\text{g/ml}$ of enopeptin A completely inhibited the viral replication, however, 100 $\mu\text{g/ml}$ of enopeptin was not enough to protect the plant from the

viral infection. After 2 days, the spots increased as same as the control (Fig. 9).

Enopeptins are a unique compound which showed a potent antiphage activity, however, because of the poor membrane permeability, the efficacy of enopeptins on animal and plant viruses was not sufficient. Therefore, improvement of the membrane permeability of the inhibitors are now undertaken.

Factumycin

Factumycin itself was already reported as an antibacterial compound¹⁰). However, in the course of our screening, we identified factumycin as an antiphage antibiotic. The pattern of

Fig. 6. The schematic model of the mode of action of RK-1441 A and B. RK-1441 A (●) showed inhibitory activity *in vitro* and *in vivo*. RK-1441 B (▲) was inactive *in vitro*, however, RK-1441 B was converted to active form (■) in the host organism and showed inhibitory activity *in vivo*.

Fig. 7. The isolation procedure of enopeptins A and B.

Table 3. Physico-chemical properties of enopeptins A and B.

	Enopeptin A	Enopeptin B
Appearance :	orange powder	orange powder
MP (°C) :	203 ~210	182~190
$[\alpha]_D^{22}$: (c 0.45, CHCl ₃)	+ 270°	+ 211°
MF :	C ₄₇ H ₅₇ N ₇ O ₁₁	C ₄₆ H ₅₅ N ₇ O ₁₁
SI-MS (m/z) :	896 (MH ⁺)	882 (MH ⁺)
HR-FAB-MS (m/z) :	896.4193 (MH ⁺)	882.4048 (MH ⁺)
calcd :	896.4195	882.4038
UV max nm(ε) :	387 (43,860)	387 (40,530)
(1%-dioxane-H ₂ O)	369 (49,230)	369 (45,500)
	354 (sh, 37,320)	354 (sh, 33,480)
	257 (16,110)	257(15,420)

Table 4. Inhibition of bacteriophage growth by enopeptin.

System	Minimal inhibition dose ^a			
	A		B	
	Phage ^b	Host ^c	Phage	Host
Enopeptin A	12.5	>200	>200	>200
Neothramycin	12.5	>200	>200	>200
RK-1441A	25	>400	50	>400

System A : phage B / *Streptomyces griseus*. System B : T4 phage / *E. coli* AB1157. a: In the assay systems, the modified agar-plate/paper-disk method was used to measure the minimal inhibition dose (μg / disk) for bacteria and bacteriophages. b: Minimal inhibition dose against the bacteriophage growth. c: Minimal inhibition dose against host bacteria.

Enopeptin A $R = CH_3$

Enopeptin B $R = H$

Fig. 8. Structures of enopeptins A and B.

Fig. 9. The protective effect of enopeptin A on *Nicotiana* leaves from the infection of tobacco mosaic viruses. *Nicotiana* leaves infected with tobacco mosaic viruses were treated with 100 µg/ml (lined column) or 500 µg/ml (filled column) of enopeptin A. Open column indicates the control without the treatment. Number of lesion was counted 1 day and 2 days after the viral infection.

antiphage activity of factumycin resembled that of tautomycin which was isolated as a potent protein phosphatase inhibitor in our laboratory. Thus, we are now investigating the inhibitory

action of factumycin on protein phosphatases.

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