CONTROLLED BIOSYNTHESIS OF NEOVIRIDOGRISEINS, NEW HOMOLOGUES OF VIRIDOGRISEIN

IV. *IN VITRO* SYNERGISM BETWEEN NEOVIRIDOGRISEIN II AND THE ANTIBIOTICS OF THE MIKAMYCIN A GROUP

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Neoviridogrisein II is a homologue of viridogrisein in which the hydroxyproline residue is replaced by proline. Neoviridogrisein II proved to be more active than the parent antibiotic against Gram-positive bacteria and *Mycoplasma* species. When neoviridogrisein II or viridogrisein was combined with griseoviridin, a non-peptidyl macrocyclic lactone, synergism was observed: maximum synergistic effect was observed for a combination ratio that depended on the test bacterium used. Neoviridogrisein II also exerted synergism when combined with mikamycin A and A-2315A.

Neoviridogriseins I, II and III are produced by a strain of *Streptomyces griseoviridus*[1]* under amino acid-controlled fermentation[2,3]; they represent the first homologues of the mikamycin B II group[4].

Viridogrisein and neoviridogriseins, as antibiotics of the mikamycin B II group, are produced together with griseoviridin, a non-peptidyl macrocyclic lactone. Synergism between mikamycin B and mikamycin A groups of antibiotics is well documented[4,5] and is useful clinically.

This paper deals with the antimicrobial activity of neoviridogriseins I, II and III and the synergism between neoviridogrisein II and griseoviridin[6], mikamycin A[7] and A-2315A[8].

Materials and Methods

Antibiotics and Reagents

Neoviridogriseins I, II and III, viridogrisein and griseoviridin were isolated from the broth of *Streptomyces* sp. P8648 by solvent extraction and column chromatography as described before[1]. Mikamycin A was obtained from Kanegafuchi Chemical Co., Ltd., Tokyo, Japan. A-2315A was kindly supplied by Dr. R. L. HAMILL, Eli Lilly and Co., Indianapolis, Ind., U.S.A. Brain heart infusion broth (BHI) and PPLO broth (CHANOCK's medium) were obtained from Difco Laboratories, Detroit, Mich., U.S.A. Other materials were purchased from commercial sources.

Microorganisms

Staphylococcus aureus FDA 209P, *S. aureus* Smith, *Sarcina lutea* S-19, *Bacillus subtilis* ATCC 6633, and *Salmonella gallinarum* ATCC 9184 were stock cultures in our laboratories. *Streptococcus pneumoniae* Type III, *Streptococcus pyogenes* NY-5, *Mycoplasma pulmonis* PG-22, *M. fermentans* and *M. agalactiae* PG-2 were kindly supplied by the Institute of Medical Science, University of Tokyo,

* *Streptomyces griseoviridis* ANDERSON, EHRLICH, SUN and BURKHOLDER (8th Ed., BERGEY's Manual of Determinative Bacteriology, The Williams & Wilkins Company, Baltimore, 1974).

Tokyo, Japan; resistant strains of *S. aureus* by Dr. H. KAWAGUCHI, Bristol-Banyu Research Laboratories Co., Tokyo, Japan; and *M. gallisepticum* KP-13 by Dr. T. OKUDA, Tanabe Seiyaku Co., Tokyo, Japan.

Determination of minimum inhibitory concentration (MIC) against bacteria

Test antibiotics were dissolved in a small amount of methanol, suitably diluted with distilled water and filter-sterilized through Millipore filter (0.22 μm, Cat. No. GSWP 01300). The sterile antibiotic solutions were diluted in BHI broth to the indicated concentrations. Serial two-fold (if necessary 2-, $\sqrt{2}$- and $\sqrt[3]{2}$-fold) dilutions of each antibiotic were made in BHI broth and distributed in 0.5 ml amounts into small sterile test tubes. Tubes were inoculated with test microorganisms (approximately 10^6 viable cells/ml) and incubated at 37°C for 16 hours. The lowest concentration of antibiotic which prevented the visually detectable growth of the test organism was defined as the minimum inhibitory concentration (MIC).

Determination of MIC against mycoplasmas

The medium was PPLO broth (pH 7.8) which contained 1% glucose and 0.002% phenol red (PR) as growth indicator. Antibiotic solutions were prepared as above except that the dilution medium was PPLO broth. Mycoplasmas were inoculated at 10^7 colony forming units/ml (CFU). Incubation was carried out at 37°C for 48 hours. The lowest concentration of antibiotic which prevented the color change of the medium was defined as MIC.

Synergism

Neoviridogrisein II and viridogrisein were combined in varying ratios with griseoviridin, mikamycin A and A-2315A and diluted in 2, $\sqrt{2}$ and $\sqrt[3]{2}$ series. The subsequent procedure of MIC determination was the same as described above. The MIC in the synergism test was defined as the smallest total amount of a combination of neoviridogrisein II or viridogrisein with griseoviridin, mikamycin A or A-2315A which inhibited the growth of the test organism.

Results

1. MIC of Neoviridogriseins I, II and III and Viridogrisein

The MIC values of neoviridogriseins I, II and III and viridogrisein on Gram-positive and Gram-negative bacteria and yeast have already been reported (Table 4 in Reference 3). Neoviridogriseins I and II looked slightly more active than neoviridogrisein III and viridogrisein against Gram-positive bacteria. No activity was recorded against the Gram-negative bacteria tested. Accordingly, the MIC values of neoviridogrisein II and viridogrisein were precisely compared, utilizing serial 2-, $\sqrt{2}$- and $\sqrt[3]{2}$-fold dilutions. As the available supply of neoviridogriseins I and III was small, only neoviridogrisein II was employed in subsequent experiments. The results are presented in Table 1.

It is clear from Table 1 that neoviridogrisein II possesses higher antibacterial activity than viridogrisein.

Neoviridogrisein II was also found more active than viridogrisein and griseoviridin against three of the four species of *Mycoplasma* tested (Table 2).

2. Synergism between Neoviridogrisein II and Antibiotics of the Mikamycin A Group

The synergistic effects of neoviridogrisein II and viridogrisein in combination with mikamycin A antibiotics were compared. The results are shown in Table 3 and Fig. 1.

The ratio that afforded highest synergism was 5: 5 (griseoviridin: neoviridogrisein II and griseoviridin: viridogrisein) when *S. lutea* S-19, *S. pneumoniae* Type-III and *M. agalactiae* PG-2 were the test organisms. For *S. aureus* FDA 209P, this ratio was 2: 8 to 1: 9. The latter ratio is not surprising, considering that griseoviridin is about 50 times less active than the depsipeptide component

Table 1. Precise comparison of neoviridogrisein II with viridogrisein in activity against Gram-positive bacteria (serial $\sqrt{2}$ and $\sqrt[3]{2}$ dilution).

Microorganism		Medium	MIC (μg/ml)	
			NVG II*	VG**
Staphylococcus aureus	FDA 209P	(1)	0.094	0.156
	(EM, CM, SM, PC, TC)^r	(1)	0.125	0.334
	(TC, CP, PC)^r	(1)	0.094	0.334
	BX-1633(PC)^r	(1)	0.125	0.267
	Russell(PC)^r	(1)	0.125	0.267
	Smith	(1)	0.125	0.267
Bacillus subtilis ATCC6633		(1)	0.094	0.250
Streptococcus pneumoniae Type-III		(2)	0.156	0.250
Streptococcus pyogenes NY-5		(2)	0.094	0.375

Medium (1): Brain heart infusion broth (pH 7.0).
(2): Brain heart infusion broth containing 10% horse blood (pH 7.0).
NVG II*: neoviridogrisein II; VG**: viridogrisein.
( )^r: antibiotic resistance to EM: erythromycin, CM: chloramphenicol, SM: streptomycin, PC: penicillin G, TC: tetracycline.
Inoculum size: 10^6 cells/ml

Table 2. MIC of neoviridogrisein II, viridogrisein and griseoviridin against species of *Mycoplasma* (serial 2-fold dilution).

Microorganism	Medium	MIC (μg/ml)		
		NVG II*	VG**	GV***
Mycoplasma gallisepticum KP-13	(1)	0.025	0.10	0.10
M. pulmonis PG-22	(2)	0.78	1.56	6.25
M. fermentans	(2)	0.05	0.05	0.05
M. agalactiae PG-2	(2)	0.39	0.78	3.13

Medium (1): PPLO enrichment broth.
(2): PPLO broth (CHANOCK's medium).
NVG II*: neoviridogrisein II; VG**: viridogrisein; GV***: griseoviridin.
Inoculum size: 10^7 CFU/ml

in the mixture.

Mikamycin A and A-2315A, which are produced by *Streptomyces mitakaensis*[9] and *Actinoplanes phillipinensis* NRRL 5462[8] respectively, also belong to the mikamycin A group of antibiotics. Mikamycin A (ostreogrycin A) was reported to show synergism with viridogrisein[10]. Therefore, the synergistic relation between neoviridogrisein II and mikamycin A or A-2315A was examined using *S. aureus* FDA 209P and *S. lutea* as test bacteria. The results are shown in Tables 4 and 5. For *S. aureus*, the most synergistic ratios were 6: 4 (mikamycin A: neoviridogrisein II) and 3: 7 (A-2315A: neoviridogrisein II). For *S. lutea* they were 5: 5 and 7: 3 respectively. Neoviridogrisein II is more active against *S. aureus* and less active against *S. lutea* than mikamycin A and A-2315A.

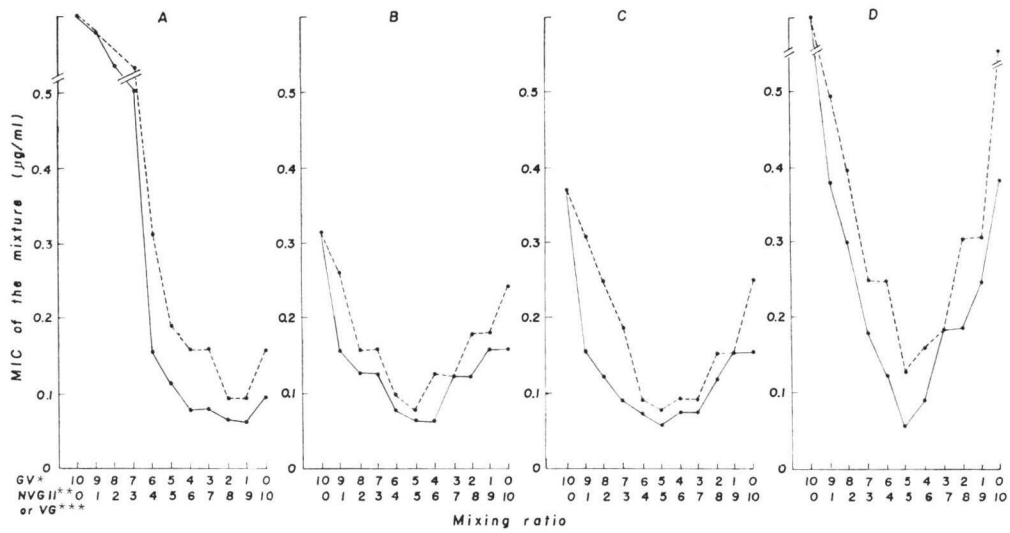
Thus, neoviridogrisein II and viridogrisein were combined with mikamycin A and the synergism of both combinations was compared, using *S. pyogenes* NY-5 and *S. aureus* Smith as test bacteria, and dilution series of $\sqrt{2}$ and $\sqrt[3]{2}$. Table 6 shows the results. For *S. pyogenes* NY-5, the

Table 3. Synergism between neoviridogrisein II or viridogrisein and griseoviridin (serial $\sqrt{2}$ and $\sqrt[3]{2}$ dilution).

Mixing ratio GV*/NVG II** or VG*** (by weight)	MIC of the mixture (μg/ml)							
	S. aureus FDA 209P		*S. lutea*		*S. pneumoniae* Type-III		*M. agalactiae* PG-2	
	NVG II	VG	NVG II	VG	NVG II	VG	NVG II	VG
10 : 0	5.0	5.0	0.313	0.313	0.375	0.375	4.0	4.0
9 : 1	1.0	1.0	0.156	0.250	0.156	0.313	0.38	0.50
8 : 2	0.75	0.75	0.125	0.156	0.125	0.250	0.31	0.38
7 : 3	0.50	0.625	0.125	0.156	0.094	0.188	0.19	0.25
6 : 4	0.157	0.313	0.078	0.094	0.078	0.094	0.13	0.25
5 : 5	0.125	0.188	0.063	0.078	0.063	0.078	0.06	0.13
4 : 6	0.079	0.157	0.063	0.125	0.078	0.094	0.09	0.16
3 : 7	0.079	0.157	0.125	0.125	0.078	0.094	0.19	0.19
2 : 8	0.063	0.094	0.125	0.188	0.125	0.156	0.19	0.31
1 : 9	0.063	0.094	0.156	0.188	0.156	0.156	0.25	0.31
0 : 10	0.094	0.156	0.156	0.250	0.156	0.250	0.39	0.78

GV*: griseoviridin; NVG II**: neoviridogrisein II; VG***: viridogrisein.

Fig. 1. Synergism between neoviridogrisein II or viridogrisein and griseoviridin.
A: *Staphylococcus aureus* FDA 209P, B: *Sarcina lutea* S-19, C: *Streptococcus pneumoniae* Type-III. D: *Mycoplasma agalactiae* PG-2.
GV*: griseoviridin, NVG II**: neoviridogrisein II, VG***: viridogrisein
●—●: griseoviridin/neoviridogrisein II, ●---●: griseoviridin/viridogrisein.



most synergistic ratios were 7: 3 to 6: 4 (neoviridogrisein II: mikamycin A) and 5: 5 (viridogrisein: mikamycin A) respectively. For *S. aureus* Smith, the most synergistic ratios were 5: 5 (neoviridogrisein II: mikamycin A and viridogrisein: mikamycin A).

It can be concluded that mikamycin A and A-2315A, as well as griseoviridin, exert similar synergy with neoviridogrisein II and viridogrisein.

Table 4. Synergism between neoviridogrisein II and mikamycin A (serial $\sqrt{2}$ and $\sqrt[3]{2}$ dilution).

Mixing ratio MK-A*/ NVG II** (by weight)	MIC of the mixture (μg/ml)	
	S. aureus FDA 209P	*S. lutea*
10 : 0	0.313	0.063
9 : 1	0.078	0.031
8 : 2	0.039	0.031
7 : 3	0.031	0.031
6 : 4	0.023	0.020
5 : 5	0.031	0.012
4 : 6	0.047	0.016
3 : 7	0.063	0.031
2 : 8	0.078	0.047
1 : 9	0.078	0.063
0 : 10	0.094	0.156

MK-A*: mikamycin A; NVG II**: neoviridogrisein II.
Medium: Brain heart infusion broth (pH 7.0).
Inoculum size: 10^6 cells/ml

Table 5. Synergism between neoviridogrisein II and A-2315A (serial $\sqrt{2}$ and $\sqrt[3]{2}$ dilution).

Mixing ratio A-2315A/ NVG II* (by weight)	MIC of the mixture (μg/ml)	
	S. aureus FDA 209P	*S. lutea*
10 : 0	0.50	0.039
9 : 1	0.25	0.039
8 : 2	0.25	0.023
7 : 3	0.25	0.0078
6 : 4	0.125	0.031
5 : 5	0.125	0.031
4 : 6	0.078	0.063
3 : 7	0.063	0.063
2 : 8	0.078	0.063
1 : 9	0.078	0.078
0 : 10	0.094	0.156

NVG II*: neoviridogrisein II.
Medium: Brain heart infusion broth (pH 7.0).
Inoculum size: 10^6 cells/ml

Table 6. Synergism between neoviridogrisein II or viridogrisein and mikamycin A (serial $\sqrt{2}$ and $\sqrt[3]{2}$ dilution).

Mixing ratio MK-A*/NVG II* or VG*** (by weight)	MIC of the mixture (μg/ml)			
	S. pyogenes NY-5		*S. aureus* Smith	
	NVG II**	VG***	NVG II**	VG***
10 : 0	0.375	0.375	2.0	2.0
9 : 1	0.125	0.188	ND†	ND
8 : 2	0.094	0.188	ND	ND
7.5 : 2.5	ND	ND	0.125	0.156
7 : 3	0.031	0.125	ND	ND
6 : 4	0.031	0.094	ND	ND
5 : 5	0.039	0.063	0.063	0.125
4 : 6	0.047	0.094	ND	ND
3 : 7	0.047	0.125	ND	ND
2.5 : 7.5	ND	ND	0.078	0.125
2 : 8	0.063	0.125	ND	ND
1 : 9	0.078	0.250	ND	ND
0 : 10	0.094	0.375	0.125	0.267

MK-A*: mikamycin A; NVG II**: neoviridogrisein II; VG***: viridogrisein
ND†: not determined.
Medium: Brain heart infusion broth (pH 7.0) for *S. aureus* Smith; brain heart infusion broth containing 10% horse blood (pH 7.0) for *S. pyogenes* NY-5.
Inoculum size: 10^6 cells/ml

Discussion

It is well known that the mikamycin B group of antibiotics exhibits a high degree of synergism

with the mikamycin A group of antibiotics[4], and this synergistic property of the mikamycin group of antibiotics has effectively been utilized in veterinary preparations.

ENGLISH *et al.*[11] reported that the (antimicrobial) activity produced by the several combinations of PA 114A and B was greater than that produced by each component singly. The best ratio was found to be 2: 8~8: 2 (A/B) for *Micrococcus pyogenes* var. *aureus*, *Bacillus subtilis* and *Mycobacterium* sp. DIJCK *et al.*[12] reported maximum synergistic effect for 70% of staphylomycin M_I and 30% of staphylomycin S, using *S. lutea* and *M. pyogenes* ATCC 65384 as test organisms. From these data and the results of the present paper, it is probable that the best ratio of mikamycin A and B groups of antibiotics depends on the test strain and the MIC values of the individual components.

Contrary to the results of WATANABE[13] in which a weak synergistic relation of mikamycin A and viridogrisein was shown by the paper cross method, our data (Tables 4 and 6) revealed that there was a marked synergism between viridogrisein or neoviridogrisein II and mikamycin A, probably because of the high sensitivity of the assay method used. Compound A-2315A which has recently been isolated from the culture broth of *Actinoplanes phillipinensis* NRRL 5462[8] was found as active as griseoviridin in synergism with neoviridogrisein II and viridogrisein.

As described in a previous paper[2], the exogenous addition of L-proline during fermentation of *Streptomyces* sp. P8648 provided neoviridogrisein II in which the *allo*-hydroxy-D-proline moiety of viridogrisein was replaced by D-proline. The absence of the hydroxyl group on the proline moiety leads to the enhancement of the antimicrobial activity. This observation has previously been shown for the actinomycins[14].

It is evident from Tables 3 and 6 and Fig. 1 that neoviridogrisein II was always more active than viridogrisein. In addition, neoviridogrisein II afforded synergism even with mikamycin A and compound A-2315A (Tables 4, 5 and 6).

Nowadays, the increasing frequency of clinically resistant pathogens is one of the serious problems encountered in the chemotherapy of infections, and, to reduce this tendency, it has been recommended that major antibiotics for human uses are prohibited from their application in animals. Though the mikamycin group of antibiotics are not useful in human therapy on account of poor solubility and absorption, they have been utilized effectively in animals as therapeutic agents and growth promoters. As neoviridogrisein II is more active than viridogrisein (and probably other mikamycin B group of antibiotics) against Gram-positive bacteria and mycoplasmas, its combination with griseoviridin, mikamycin A or compound A-2315A is expected to be valuable in veterinary applications.

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