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Effects of Griseoviridin and Viridogrisein against Swine Dysentery in Experimental Infection by Using Mice and Pigs

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ABSTRACT. Griseoviridin, a known antibiotic produced by *Streptomyces cacaoi* subsp. *cacaoi*, was found to be active against *Brachyspira hyodysenteriae*—the bacterium causing swine dysentery. An *in vitro* synergism is observed when it is used in combination with viridogrisein—a simultaneously produced antibiotic. In mouse experiments, the effect of griseoviridin alone was less than that of lincomycin—a commercially available swine dysentery medication. However, a 1:1 mixture of griseoviridin and viridogrisein revealed a noticeable synergistic effect. In an evaluation using pigs artificially infected with *B. hyodysenteriae*, a large difference was not observed between the effect of griseoviridin alone and that in combination with viridogrisein. Nevertheless, griseoviridin alone exhibited a therapeutic effect superior to that of lincomycin.

KEY WORDS: *Brachyspira hyodysenteriae*, griseoviridin, swine dysentery, viridogrisein.

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Swine dysentery is a chronic or fatal contagious gastrointestinal disease characterized by mucohemorrhagic diarrhea, and it is primarily caused by *Brachyspira hyodysenteriae* [14]. This pathogen is present in a large number in the feces of infected pigs, and the breeding environment is rapidly contaminated through oral infection. The number of animals as well as the practice of breeding in groups has increased in the pig industry, thereby increasing the damage from the spread of swine dysentery. Thus far, a variety of chemotherapeutic drugs and antibiotics have been developed for the prevention and treatment of swine dysentery. Carbadox has been used as a synthetic antimicrobial agent, and tiamulin, sedecamycin, lincomycin, and efrotomycin have been used as antibiotics; however, the appearance of resistant bacteria has become an issue of concern [5, 8, 15].

On the other hand, it was reported that the known antibiotics griseoviridin and viridogrisein were simultaneously produced by some strains of streptomycetes [1], and their chemical structures were similar to those of the animal growth promoters virginiamycin M and S, respectively [2]. It has also been reported that viridogrisein has three minor homologues, namely, neoviridogriseins I, II, and III [10, 11].

We detected antimicrobial activity in a streptomyces fermentation product containing both griseoviridin and viridogrisein. Furthermore, it was confirmed that even griseoviridin alone exhibited an *in vitro* antimicrobial activity and synergism occurred when it was used in combination with viridogrisein. In this paper, we report the effects of these antibiotics against swine dysentery in an experimental infection by using mice and pigs.

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MATERIALS AND METHODS

Fermentative production and purification of griseoviridin and viridogrisein: The antibiotic-producing stock strain of *Streptomyces cacaoi* subsp. *cacaoi* No. 7505 was inoculated into a culture medium (maltodextrin, 2.0%; peanut powder, 1.0%; corn steep liquor, 1.0%; $CaCO_3$, 0.2% (pH: 6.5)) and then incubated for 5~6 days at 30°C with stirring to entrain air. The culture fluid was filtered and adjusted to a pH of 6.0, and the active substances were extracted using ethyl acetate. After concentrating the extract, fractionation and purification were performed using column chromatography through a Silicagel column (Silicagel 60, Merck, U.S.A.). Viridogrisein crystals were obtained by adding acetone saturated with hydrogen chloride into the concentrated elution fraction, and griseoviridin crystals were obtained by dissolving the elution fraction in a small amount of methanol and then allowing it to concentrate.

Griseoviridin and viridogrisein (etamycin or neoviridogrisein IV) are known antimicrobials [1, 10, 11]; their molecular structures have been elucidated (Fig. 1).

Measurement of minimum inhibitory concentration (MIC) in vitro against B. hyodysenteriae: The MIC values of the antimicrobial compounds against *B. hyodysenteriae* were determined by the agar dilution method by a twofold dilution from 100 to 0.1 μg/m*l* final concentration of the compounds in Trypticase soy agar (BBL, U.S.A.) containing 5% sheep blood, as described by Kitai *et al.* [6]. Lincomycin (Sigma, U.S.A.) and virginiamycin (Sigma, U.S.A.) were used as reference drugs.

Evaluation of the inhibitory effect of the antimicrobials on infection with B. hyodysenteriae in mice: Five-week-old mice (ddY, male) were used, and 100 ppm spectinomycin was administered in drinking water, starting 3 days before infection until the conclusion of the experiment. The mice

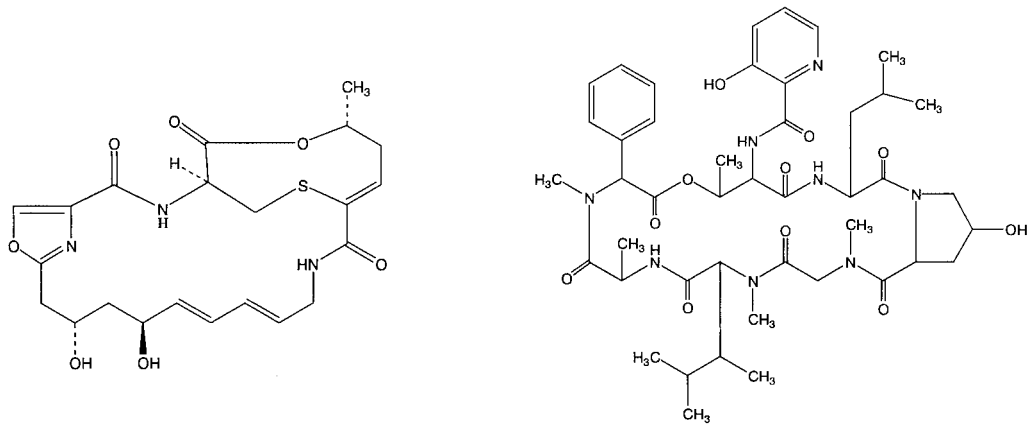

Fig. 1. Chemical structural formulae of griseoviridin and viridogrisein.

were orally infected with approximately 10^7 cells of *B. hyodysenteriae* ATCC31212 suspended in 0.1 M phosphate buffered saline. On the first and second day after infection, the solutions of the test compounds (20, 40, and 80 mg/kg of griseoviridin; 40 and 80 mg/kg of viridogrisein; 1.25, 2.5, and 5.0 mg/kg of 1:1 mixture of griseoviridin:viridogrisein; 20, 40, and 80 mg/kg of lincomycin; and 100 mg/kg of virginiamycin) were administered orally once per day. On the fourth day after infection, the mice were killed with CO_2. The cecum from each mouse was removed and examined for lesions (organ atrophy, intraluminal mucus, hemorrhage, etc.). The following swab method was used to compare the number of *B. hyodysenteriae* in the contents of the cecum. A cotton swab was used to wipe the contents from the cecum and then transfer them onto an agar plate of CVS medium (Trypticase soy broth (BBL, U.S.A.) supplemented with 5% sheep blood and containing spectinomycin, 400 μg/m*l*; colistin, 25 μg/m*l*; and vancomycin, 25 μg/m*l* as selective drugs) with 1.2% agar. The agar plates were then anaerobically incubated at 42°C for 72 hr with a GasPak system (BBL, U.S.A.). A group of four mice was used for each evaluation study. The number of mice showing cecal lesions as well as the number of mice showing the presence of *B. hyodysenteriae* in the cecum in the swab method was compared among the groups.

Evaluation of the therapeutic effect in pigs infected with B. hyodysenteriae: Specific pathogen free (SPF) pigs (Landrace × Large White hybrid, male) weighing approximately 8 kg were used. The animals were kept in stainless steel cages (90 × 90 cm) with raised plastic netting floors. Two pigs were kept in each cage and were allowed to freely ingest water and feed (substitute milk for piglets, antibacterial-free, for the second half, Nippon Formula Feed Mfg.). *B. hyodysenteriae* ATCC31212 was inoculated into Trypticase soy broth supplemented with 5% sheep blood. The liquid medium was divided into 10-m*l* samples and poured into petri dishes (diameter, 9 cm) and anaerobically incubated for 2–3 days at 42°C using a GasPak system. This precultured fluid was inoculated into a fresh liquid medium at a concentration of 5% and cultured under the same conditions, resulting in a large amount of bacterial culture that is infectious to swine.

Pigs were fasted for 18 hr prior to infection. Each animal was artificially inoculated with 100 m*l* of the *B. hyodysenteriae* culture fluid twice a day (total of 200 m*l*) via an intragastric catheter. Each animal was inoculated with 10^9–10^{10} cells/200 m*l* of bacteria. Clinical symptoms of swine dysentery (watery and/or mucohemorrhagic diarrhea) started appearing from the third day to the second week after inoculation; most of the animals showed the symptoms approximately 1 week after the inoculation. Samples of the test compounds were administered to the pigs that showed no improvements in the symptoms 2 to 4 days after the onset of the symptoms. Two dosing methods were used. One method involved forceful administration via intragastric catheter at the rate of one dose (0.1 and 1.0 mg/kg of griseoviridin; 0.1 and 1.0 mg/kg of 1:1 mixture of griseoviridin:viridogrisein; and 0.1 and 1.25 mg/kg of lincomycin) per day for a period of 2 days (forced oral dosing). In the other method, a fixed concentration of the compound (2.5 and 5.0 ppm of griseoviridin; 5.0 ppm of lincomycin; and 50 ppm of virginiamycin) was mixed into the feed that was made freely available for a period of 7 days (feed additive dosing). For 7 days after the start of dosing, the body weights of the pigs were measured, the clinical signs and feces condition were examined, and the *B. hyodysenteriae* bacteria counts in the feces were estimated.

The following method was used for estimating the bacterial counts in the feces. The CVS medium containing 1.0% agar was prepared and kept warm to prevent solidification. Feces were serially diluted (10-fold dilutions) with 0.1 M phosphate buffered saline, and 1 m*l* of the diluted fluids was mixed with 9 m*l* of the above-mentioned selective medium. The agar plates were prepared by allowing the mixture to

solidify. The inoculated plates were then anaerobically incubated at 42°C for 3 days. After the incubation, the beta hemolytic colonies were counted, and the number of bacteria was expressed as the $\log_{10}$ of colony-forming units per gram of fecal matter (CFU/g). On the seventh day after the start of dosing, the test pigs were killed with CO_2 and bled. The colons were removed and examined for lesions (thickened colonic wall, mucosal edema, mucosal hemorrhage, etc.).

RESULTS

In vitro antibacterial activity of griseoviridin and viridogrisein against B. hyodysenteriae: The MIC of griseoviridin, viridogrisein, and a 1:1 mixture of both drugs against *B. hyodysenteriae* was measured. The results are shown in Table 1. Among the bacterial stock cultures used, S73/2 and S57081 show susceptibility to macrolide-type antimicrobials while the remaining three stocks are resistant to macrolides. The MIC of lincomycin also shows a large difference between the resistant strains and the susceptible strains. A comparison of the MIC of griseoviridin and viridogrisein indicates that although there were differences depending on the bacterial strain and that both were antibiotically active against *B. hyodysenteria*e, griseoviridin had a stronger activity. In particular, griseoviridin showed an eightfold greater activity than viridogrisein against the ATCC31212 strain (macrolide-resistant strain) used for the *in vivo* evaluation. Furthermore, griseoviridin showed MIC values similar to those of virginiamycin, which has a similar chemical structure. The activity of the 1:1 mixture of griseoviridin and viridogrisein (G + V) was clearly stronger than that of either of the components alone, thus indicating a synergistic effect.

In vivo inhibitory effects of griseoviridin and viridogrisein on B. hyodysenteriae infection in mice: The effects of griseoviridin, viridogrisein, and a 1:1 mixture of the two compounds on infected mice were compared with those of the known swine dysentery therapeutic agent lincomycin. The results are shown in Table 2. The effects of griseoviridin and viridogrisein alone were less than those of lincomycin; the comparison was made depending on the number of mice from which bacteria were isolated in each group administered with drugs at a dose of 40 or 80 mg/kg/day × 2. However, the combination of griseoviridin and viridogrisein in a 1:1 proportion exhibited a synergistic effect remarkably greater than that of the in vitro activity. Furthermore, in the case of the *in vitro* activity, virginiamycin exhibited MIC values similar to those of griseoviridin; however, no effect was observed in the *in vivo* evaluation in

Table 1. MIC (μg/m*l*) of griseoviridin and viridogrisein against *Brachyspira hyodysenteriae*

Strain	Griseoviridin	Viridogrisein	G + V (1:1)[a)]	Lincomycin	Virginiamycin
ATCC31212	1.56	12.5	0.39	25	1.56
DJ 70	0.2	12.5	0.2	12.5	0.78
S-5	0.78	6.25	0.2	25	1.56
S73/2	0.78	3.13	0.39	0.39	0.78
S57081	1.56	3.13	0.78	0.39	1.56

a) 1:1 mixture of griseoviridin: viridogrisein.

Table 2. Inhibitory effects of griseoviridin and viridogrisein on *B. hyodysenteriae* ATCC31212 infection in mice

Test group	Dosage (mg/kg/day × 2)	Cecal lesion	Bacteria isolated from the cecum
		Number of mice with lesions	Number of mice from which bacteria were isolated
Undosed control	–	4/4	4/4
Griseoviridin	20	0/4	4/4
	40	0/4	4/4
	80	0/4	2/4
Viridogrisein	40	2/4	4/4
	80	0/4	4/4
G + V (1:1)[a)]	1.25	0/4	4/4
	2.5	0/4	3/4
	5	0/4	0/4
Lincomycin	20	0/4	4/4
	40	0/4	1/4
	80	0/4	0/4
Virginiamycin	100	4/4	4/4

a) 1:1 mixture of griseoviridin: viridogrisein.

mice.

Therapeutic effects of griseoviridin and viridogrisein on infection with B. hyodysenteriae in pigs: The therapeutic effects of griseoviridin alone, a 1:1 mixture of griseoviridin and viridogrisein, lincomycin and virginiamycin administered to pigs infected with *B. hyodysenteriae* were compared. The results are shown in Table 3. In the treatments involving forced oral dosing of griseoviridin alone or a 1:1 mixture of griseoviridin and viridogrisein, one of the two pigs that received doses of 0.1 mg/kg × 2 recovered, while all the pigs receiving doses of 1.0 mg/kg × 2 recovered. Unlike mice, no synergistic effect was observed in pigs when the effect of griseoviridin was compared with that of the mixture combined with viridogrisein. No effect was observed in pigs receiving doses of 0.1 mg/kg × 2 of lincomycin, and one of the two pigs that received doses of 1.25 mg/kg × 2 doses recovered. In the treatments via feed additive dosing, feces of both pigs in the griseoviridin 2.5-ppm dosage group became normal; however, in one pig, the bacteria did not completely disappear from the feces. In the 5.0-ppm dosage group, both pigs recovered. An improvement was observed in the symptoms in the lincomycin 5.0-ppm group, but neither of the two pigs recovered. Virginiamycin exhibited no inhibitory effects even at a dosage of 50 ppm.

DISCUSSION

We have investigated many antibiotics and antimicrobial agents for their inhibitory effects, including *in vitro* activity, effects of the drugs in mice, and clinical effects in pigs, but the correlation between these studies could not be confirmed. For example, the MIC values of penicillin G (penicillin group), furazolidone (nitrofuran group), and monensin (polyether group) obtained in *in vitro* studies are less than 1.0 (μg/m*l*) [7, 15]; however, none of them are expected to have a preventive or therapeutic effect against swine dysentery. The MIC value for lincomycin is 25–100 μg/m*l* and is marketed as having excellent preventive and therapeutic effects against swine dysentery.

The effects of griseoviridin and viridogrisein alone in the mouse experiments were less than those of lincomycin. However, the 1:1 mixture of griseoviridin and viridogrisein revealed a noticeable synergistic effect. Therefore, an actual experiment was conducted on pigs artificially infected with *B. hyodysenteriae*. However, no large difference was observed between the effect of griseoviridin alone and that of a combination of griseoviridin and viridogrisein. Nevertheless, in the evaluation using pigs, the results were in contrast with those from the experiments on mice. Griseoviridin alone was shown to exhibit a therapeutic effect superior to that of lincomycin. On the other hand, the MIC values of virginiamycin M, which is structurally similar to griseoviridin [2] and has been used as an animal growth promoter, obtained in *in vitro* studies are similar to those of griseoviridin. However, in the therapeutic effect tests on swine dysentery using the doses added to the feed, griseoviridin showed an effect at a dose of 2.5 ppm, but virginiamycin [12] did not show any effect even at a dose of 50 ppm.

Significant differences were found between the *in vitro* activity and the *in vivo* effects. A reason for the lack of an apparent correlation may be that the complex intestinal flora significantly affects an infectious intestinal disease such as swine dysentery. It is highly probable that the other intestinal flora contribute to the onset of symptoms. For instance, it was reported that clinical symptoms of swine dysentery were induced in gnotobiotic pigs inoculated with *B. hyodysenteriae* in combination with two other anaerobes, namely, *Fusobacterium necrophorum* and *Bacteroides vulgatus*, although no symptoms were induced when inoculated with *B. hyodysenteriae* alone [3, 16].

Similarly, it has been reported that the infection of mice with *B. hyodysenteriae* is influenced by certain intestinal anaerobes. Hayashi *et al.* [4] reported that the high susceptibility of Ta:CF#1 mice to *B. hyodysenteriae* infection is due to the presence of *Bacteroides uniformis* in the cecal contents. In contrast, Suenaga *et al.* [13] reported that Ta:CF#1 mice, which were originally susceptible to *B. hyodysenteriae* infection, became resistant to the infection after being inoculated orally with the fecal suspensions obtained from the resistant Slc:ICR mice. Although Slc:ICR mice are not infected under normal circumstances, infection is induced by administering spectinomycin beforehand. It is believed that spectinomycin eliminates the original bacteria that prevent infection.

We thought that it might be possible to easily infect pigs with swine dysentery by the same pretreatment used in the experiment with mice. Therefore, 50 ppm of spectinomycin was added to the feed, and the pigs were then infected with *B. hyodysenteriae*. However, the results obtained were contradictory to the expected results. An onset of clinical symptoms was observed in untreated pigs, while the pigs administered with spectinomycin did not become symptomatic [9]. This suggests that spectinomycin may have a preventive effect on swine dysentery although it has no antibacterial activity against *B. hyodysenteriae*. The intestinal flora of mice and pigs is completely different. The bacteria necessary for the onset of symptoms of swine dysentery are present in pigs. Since these bacteria are susceptible to the effects of spectinomycin, it is possible that a preventive effect will be achieved. In the experiments on mice, a notable synergistic effect of griseoviridin and viridogrisein was observed; however, no such effect was observed in pigs. This result is also probably due to the influence of the different intestinal flora in mice and pigs.

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Table 3. Therapeutic effects of griseoviridin and viridogrisein on *B. hyodysenteriae* ATCC31212 infection in pigs

Test group (2 specimens each)	Changes in feces condition[a] and bacterial count[b] — Days after dosing								Colonic lesion[c]	7-day weight gain (kg)
	0	1	2	3	4	5	6	7		
Not infected/not dosed	–	–	–	–	–	–	–	±	–	2.0
	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2		
	–	–	–	+	–	±	–	–	–	1.5
	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2		
Infected/not dosed	++	++	++	++	++	++	++	++	+	–1.0
	7.1	8.1	7.9	7.3	7.9	7.1	7.3	6.9		
	+	+	++	++	++	++	++	++	+	–1.5
	5.7	6.4	6.7	7.5	7.9	7.6	8.3	7.3		
Griseoviridin 0.1 mg/kg × 2	++	±	–	–	–	–	–	–	–	3.0
	7.1	3.4	< 2	< 2	< 2	< 2	< 2	< 2		
	++	+	+	+	++	++	++	++	+	–0.5
	7.1	4.6	4.8	6.2	6.8	7.5	7.5	8.4		
Griseoviridin 1.0 mg/kg × 2	++	±	–	–	–	–	–	–	–	2.5
	6.3	2.5	< 2	< 2	< 2	< 2	< 2	< 2		
	++	±	–	–	–	–	–	–	–	4.0
	7.1	2.0	< 2	< 2	< 2	< 2	< 2	< 2		
G + V (1/1)[d] 0.1 mg/kg × 2	++	+	–	–	–	–	+	++	±	1.0
	7.0	3.0	< 2	2.0	2.0	< 2	3.7	4.1		
	++	+	–	–	–	–	–	–	–	5.0
	7.8	2.3	< 2	< 2	< 2	< 2	< 2	< 2		
G + V (1/1) 1.0 mg/kg × 2	++	±	–	–	–	–	–	–	–	4.5
	7.0	< 2	< 2	< 2	< 2	< 2	< 2	< 2		
	++	±	–	–	–	–	–	–	–	5.0
	7.6	5.7	< 2	< 2	< 2	< 2	< 2	< 2		
Lincomycin 0.1 mg/kg × 2	++	++	++	++	++	+	++	++	+	–0.5
	7.6	6.9	5.5	6.2	6.3	6.0	6.0	6.6		
	++	++	++	++	++	++	++	++	++	–1.0
	7.2	6.7	6.9	7.7	7.3	7.8	6.3	6.1		
Lincomycin 1.25 mg/kg × 2	++	++	±	–	–	±	±	±	–	1.0
	7.0	4.8	< 2	< 2	< 2	< 2	< 2	< 2		
	++	+	+	–	±	±	++	+	++	2.5
	6.9	< 2	4.6	3.5	5.3	5.3	7.2	6.0		
Griseoviridin 2.5 ppm	++	++	±	±	–	–	–	–	–	2.5
	7.4	6.3	7.6	6.1	4.3	< 2	< 2	< 2		
	++	+	–	–	–	–	–	–	–	4.0
	7.7	4.6	< 2	2.0	2.0	2.7	2.0	3.2		
Griseoviridin 5.0 ppm	++	++	±	±	–	–	–	–	±	0.5
	7.7	8.1	6.6	5.4	5.3	6.2	< 2	< 2		
	++	±	–	–	–	–	–	–	–	3.5
	7.6	7.3	2.7	2.8	2.0	< 2	< 2	< 2		
Lincomycin 5.0 ppm	++	+	+	++	++	++	+	+	+	0
	6.6	7.0	6.8	6.9	7.0	7.1	7.5	6.4		
	++	+	–	–	±	–	–	–	±	4.5
	7.1	4.8	< 2	6.3	4.0	3.6	4.0	2.6		
Virginiamycin 50 ppm	++	++	±	+	++	++	+	++	++	0.5
	7.7	7.0	6.9	5.3	7.3	7.8	7.1	7.1		
	++	++	++	++	++	++	++	++	++	–1.0
	8.1	7.7	7.2	5.5	7.8	7.3	7.5	8.0		

a) Feces condition (upper row): –, normal feces; ±, soft feces; +, diarrhea; ++, dysentery or watery feces with mucus.
b) Bacterial count in the feces (lower row): unit, $\log_{10}$ CFU/g; detection limit, 1×10^2 CFU/g.
c) Score of colonic lesion (thickened colonic wall, mucosal edema, mucosal hemorrhage, etc.): –, normal; ±, slight; +, moderate; ++, serious degree.
d) 1:1 mixture of griseoviridin–viridogrisein

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