PHENELFAMYCINS, A NOVEL COMPLEX OF ELFAMYCIN-TYPE ANTIBIOTICS

III. ACTIVITY *IN VITRO* AND IN A HAMSTER COLITIS MODEL

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Phenelfamycins A, B, C, E, F and unphenelfamycin make up a recently isolated group of elfamycin-type antibiotics. All of the phenelfamycins were active against Gram-positive anaerobes, including *Clostridium difficile*. Phenelfamycin A was also active *in vitro* against *Neisseria gonorrhoeae* and Streptococci. Phenelfamycin A was found to be effective in prolonging the survival of hamsters in an animal model of *C. difficile* enterocolitis. After oral administration of phenelfamycin A to hamsters, antibiotic was detected in the caecal contents but not in the blood.

Phenelfamycins are a complex of elfamycin-type antibiotics. Other antibiotics reported to have related chemical structures include efrotomycin,[1] aurodox,[2] kirromycin[3] and several other compounds.[4] The discovery, taxonomy and fermentation of the producing culture and the isolation and structure elucidation of the phenelfamycins have been described in the two preceding papers.[5,6] The present report describes the antibacterial activity of these compounds, including their *in vitro* potencies against a number of aerobic and anaerobic bacteria. In addition, the *in vivo* activity of one of these compounds, phenelfamycin A, is evaluated in a hamster model of pseudomembranous colitis caused by *Clostridium difficile*.

Materials and Methods

Bacterial Strains

Strains used in this study were from the Abbott culture collection and the American Type Culture Collection except *Neisseria gonorrhoeae* F28 which was from the Centers for Disease Control, Atlanta, Georgia. All strains were maintained frozen at −60°C.

Antibacterial Agents

The following compounds were tested and unless noted otherwise were prepared at Abbott Laboratories; phenelfamycins A, B, C, E, F, unphenelfamycin, efrotomycin, erythromycin A, vancomycin (Eli Lilly and Company, Indianapolis, Indiana), clindamycin (The Upjohn Company, Kalamazoo, Michigan) and metronidazole (G.D. Searle & Co., San Juan, Puerto Rico). The phenelfamycins and unphenelfamycin were dissolved in methanol for *in vitro* tests. For *in vivo* studies, a suspension of phenelfamycin A was prepared by addition of sterile water followed by sonification.

In Vitro Activity

MICs were determined using the standard 2-fold agar dilution procedure.† Brain heart infusion

† National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Standard M7-A. Reference agar dilution procedure for antimicrobic susceptibility testing of anaerobic bacteria. Standard M11-A. National Committee for Clinical Laboratory Standards, Villanova, Pennsylvania, 1985.

agar was used for aerobes, Wilkins-Chalgren agar was used for anaerobes and Proteose No. 3 agar (GIBCO Laboratories, Madison, Wisconsin) with hemoglobin 1% and Kellogg supplement 1% (an enrichment solution containing glucose 40%, glutamine 1%, thiamine pyrophosphate 0.002% and $Fe(NO_3)_3 \cdot 9H_2O$ 0.085%) was used for *N. gonorrhoeae*. *N. gonorrhoeae* was incubated in a 7% CO_2 atmosphere. *Candida albicans* was tested by an analogous 2-fold agar dilution method using yeast nitrogen base agar buffered at pH 7 and containing 0.5% glucose.[7]

To assess the bioavailability of unphenelfamycin in the presence of hamster caecal contents, MICs and MBCs were determined in Wilkins-Chalgren broth which had been mixed with caecal material. Caecal material (approximately 0.5 g) was suspended by vigorous vortexing into 10 ml broth containing unphenelfamycin at a concentration of 256 μg/ml. The mixture was allowed to stand at room temperature for 30 minutes followed by centrifugation at $1,000 \times g$ to pellet solid matter. The supernatant material was then filtered through gauze mesh, a 0.8-μm nitrocellulose filter and finally sterilized by passage through a 0.2-μm nitrocellulose filter. Broth containing unphenelfamycin but without caecal material was included as a control. MICs for *C. difficile* ATCC 9689 were determined by a 2-fold microdilution method.† MBCs were determined by plating 10 μl aliquots from each well showing no growth and from the well containing one-half the MIC onto drug-free agar followed by 48-hour incubation. Rejection values for determining 99.9% killing were as previously published.[8]

Time-kill Studies

The bactericidal activity of unphenelfamycin (MIC 8 μg/ml) and vancomycin (MIC 1 μg/ml) against *C. difficile* ATCC 9689 over time was determined in Wilkins-Chalgren broth by a method previously described.[9] Viability of *C. difficile* ATCC 9689 was determined at 0.5, 1, 2, 4, 6 and 24 hours after exposure to four and eight times the MIC of the compounds.

Frequency of Resistance

The frequency of spontaneous resistance development by *C. difficile* ATCC 9689 to four and eight times the MIC of unphenelfamycin was determined in Wilkins-Chalgren agar by a previously described method.[10]

Hamster Model for Pseudomembranous Colitis

The hamster colitis test used in this study has been described previously.[11,12] Male Golden Syrian hamsters, weighing 80~100 g, were purchased from Harlan Sprague Dawley, Inc., Indianapolis, Indiana. The hamsters were caged in groups of five and were given Purina Rodent Chow and water *ad libitum*. After a quarantine period of 7 days, the hamsters were exposed to *C. difficile* ATCC 9689 by addition of 10^6 bacteria per ml in their drinking water. Viable cell counts were performed immediately after the addition of *C. difficile* to drinking water. Following exposure to *C. difficile* the hamsters were immediately challenged with 100 mg/kg clindamycin by oral gavage. Beginning 8 hours after clindamycin treatment hamsters received 25 mg/kg/day vancomycin or phenelfamycin A. Both compounds were administered orally by gavage in a volume of 0.5 ml. Ten animals were used in each treatment group. Two animals from each treatment group were sacrificed 1, 4 and 7 days after clindamycin treatment. The caecal contents were removed from each of these hamsters and analyzed for *C. difficile* antigens using a rapid latex slide agglutination test (Marion Laboratories, Kansas City, Missouri). An increase in this antigen has been correlated with disease in humans.[13] The remaining four hamsters from each treatment group were monitored for 16 days and the cumulative mortality during this period was recorded. Prior to running this test, enumeration of the caecal microflora was carried out in hamsters before and after administration of clindamycin to insure that the presence of *C. difficile* antigens correlated with changes in flora known to be associated with antibiotic-induced pseudomembranous colitis. Caecal contents were weighed, then added to 10 ml prereduced thioglycolate broth on ice. Ten-fold dilutions of this suspension were cultured aerobically on Columbia sheep blood agar and MacConkey agar and anaerobically on reducible Columbia sheep blood agar with and without kanamycin and on CCFA, a selective medium containing cycloserine, cefoxitin, fructose and egg yolk agar (GIBCO).

† See footnote on p. 94.

Pharmacokinetic Studies in Hamsters

Male Golden Syrian hamsters, weighing 80~100 g, were administered 25 mg/kg phenelfamycin A orally. At 1, 2, 3 and 6 hours after administration, blood and caecal contents were collected from groups of two hamsters. Samples of serum and caecal contents were assayed for phenelfamycin A by HPLC. The HPLC methodology is described elsewhere.[6]

Results

In Vitro Activity

The MICs of phenelfamycin A and efrotomycin for a variety of aerobic organisms are shown in Table 1. Both compounds have activity against Streptococci. The MICs of phenelfamycin A for *N. gonorrhoeae* are generally the same as or within one 2-fold dilution of those of erythromycin A (Table 2). Phenelfamycins A through F and unphenelfamycin are more active against Gram-positive anaerobes than against Gram-negative anaerobes (Table 3); the phenelfamycins are more active than unphenelfamycin and as active as efrotomycin against Gram-positive anaerobes. MICs of phenelfamycins and unphenelfamycin for *C. difficile* are shown in Table 4. Phenelfamycins E and F are one to two 2-fold dilutions more active than phenelfamycin C which is generally one to two 2-fold dilutions more active than phenelfamycins A and B which are generally one to three 2-fold dilutions more active than unphenelfamycin against *C. difficile*. Phenelfamycins E and F were two to four 2-fold dilutions

Table 1. Activity of phenelfamycin A and efrotomycin against aerobic organisms.

Strain	MIC (μg/ml)	
	Phenelfamycin A	Efrotomycin
Staphylococcus aureus ATCC 6538P	>100	100
S. aureus CMX 686B	>100	>100
S. aureus A5177	>100	100
S. aureus 45	>100	>100
S. epidermidis 3519	>100	>100
Micrococcus luteus ATCC 9341	25	50
Streptococcus pyogenes EES61	3.1	6.2
S. agalactiae CMX 508	0.78	3.1
S. bovis A5169	6.2	3.1
Enterococcus faecium ATCC 8043	6.2	12.5
Escherichia coli Juhl	>100	100
Pseudomonas aeruginosa A5007	>100	100

Table 2. Activity of phenelfamycin A and erythromycin against *Neisseria gonorrhoeae*.

Strain	MIC (μg/ml)	
	Phenelfamycin A	Erythromycin A
N. gonorrhoeae CMX 556	0.5	0.5
N. gonorrhoeae CMX 557	1	1
N. gonorrhoeae CMX 558	0.5	0.5
N. gonorrhoeae CMX 591	1	0.5
N. gonorrhoeae CMX 638	1	0.25
N. gonorrhoeae CMX 664	1	1
N. gonorrhoeae 35F AMP I	0.5	0.25
N. gonorrhoeae 389 AMP R	2	1
N. gonorrhoeae F28	1	0.12

Table 3. Activity of phenelfamycins, unphenelfamycin, efrotomycin and clindamycin against anaerobes.

Strain	MIC (μg/ml)							
	A	B	C	E	F	U	EF	CL
Bacteroides fragilis ATCC 25285	>128	>128	>128	>128	>128	>128	128	1
B. fragilis 784	>128	>128	>128	>128	>128	>128	128	1
B. fragilis UC-2	>128	>128	>128	>128	>128	>128	128	0.12
B. thetaiotaomicron ATCC 29741	>128	>128	>128	>128	>128	>128	>128	4
B. melaninogenicus ATCC 25845	>128	>128	>128	64	64	>128	32	0.03
Fusobacterium nucleatum ATCC 25586	>128	32	16	64	64	64	8	0.25
Veillonella parvula ATCC 10790	16	64	64	32	16	64	32	0.06
Clostridium perfringens ATCC 13124	2	32	16	2	2	>128	2	0.06
C. perfringens 788	2	64	8	2	1	128	1	0.06
C. difficile ATCC 17857	2	4	2	0.5	0.5	16	0.5	8
C. ramosum 7	1	2	1	1	0.25	128	128	1
Propionibacterium acnes 132	0.5	8	16	1	0.25	4	16	0.06
Peptococcus asaccharolyticus ATCC 14963	0.5	0.5	0.5	0.06	0.25	16	ND	0.06
P. magnus ATCC 29328	0.5	ND	ND	0.06	0.25	32	16	1
Peptostreptococcus sp.	8	16	16	16	8	32	16	0.06
Peptostreptococcus micros ATCC 33270	0.25	ND	ND	0.12	0.03	2	0.06	0.03
P. anaerobius ATCC 27337	0.25	0.5	0.12	0.12	0.03	ND	ND	0.06

A, B, C, E and F: Phenelfamycins A, B, C, E and F, U: unphenelfamycin, EF: efrotomycin, CL: clindamycin.
ND: Not done.

Table 4. Activity of phenelfamycins A, B, C, E, F, unphenelfamycin, vancomycin, clindamycin and metronidazole against *Clostridium difficile*.

Strain	MIC (μg/ml)								
	A	B	C	E	F	U	V	CL	M
C. difficile ATCC 9689	4	4	1	0.5	0.5	16	4	4	0.25
C. difficile ATCC 17857	4	4	2	0.5	0.5	16	8	4	0.5
C. difficile 2528	2	4	1	1	0.5	16	8	4	0.5
C. difficile 2529	2	4	1	0.5	0.5	16	8	4	0.5
C. difficile 2530	8	8	2	1	0.5	16	2	4	0.25
C. difficile 2531	2	2	1	0.5	0.5	16	2	2	0.12
C. difficile 2532	4	4	2	1	0.5	16	4	4	0.12
C. difficile 2533	32	16	16	1	0.5	16	4	4	0.25
C. difficile 2535	0.5	1	1	0.5	0.25	16	8	2	0.25
C. difficile 2536	2	2	1	0.25	0.25	8	1	4	0.25
C. difficile 2537	1	2	1	0.25	0.25	8	1	4	0.25

A, B, C, E and F: Phenelfamycins A, B, C, E and F, U: unphenelfamycin, V: vancomycin, CL: clindamycin, M: metronidazole.

more active than vancomycin and clindamycin against *C. difficile*. MICs of phenelfamycin A for nine strains of *C. albicans* were >100 μg/ml.

The MIC and MBC of unphenelfamycin in the presence of hamster caecal material for *C. difficile* ATCC 9689 was 64 and 128 μg/ml, respectively, while the control MIC and MBC were 4 and 16 μg/ml, respectively.

Time-kill Studies

Viable cell counts of *C. difficile* ATCC 9689 at the beginning of time-kill studies were approximately 1.0×10^6 cfu/ml. Viable cell counts after exposure to four and eight times the MIC of unphenelfamycin remained constant at all sampling times. Viable cell counts after exposure to four and eight times the MIC of vancomycin were reduced 99% and >99.9% at 6 and 24 hours, respectively.

Frequency of Resistance

The frequency of spontaneous resistance by *C. difficile* ATCC 9689 to four and eight times the MIC of unphenelfamycin was 1.0×10^{-8} and $<1.0\times10^{-8}$, respectively.

In Vivo Activity

After administration of clindamycin all control hamsters died within 5 days. These animals were lethargic and had diarrhea prior to death. Necropsy revealed distended hemorrhagic caeca containing watery fluid. *C. difficile* antigens were isolated from the caecal contents of all control hamsters dying during the experiment. Previous testing in our laboratory indicated that the caecal microflora of hamsters dying after clindamycin administration does undergo changes consistent with antibiotic-associated enterocolitis. These changes include a marked decrease in anaerobic Gram-positive cocci and anaerobic Gram-negative bacilli along with an increase in anaerobic Gram-positive bacilli with *C. difficile* present.

Oral administration of 25 mg/kg/day phenelfamycin A for 5 days prolonged the survival of clindamycin challenged hamsters. As indicated in Fig. 1, phenelfamycin A treated hamsters lived up to 7 days longer than controls. Hamsters receiving an equal dose of vancomycin lived up to 11 days longer than controls. The mean-time-to-death in animals receiving vancomycin, phenelfamycin A or no

Fig. 1. Cumulative mortality due to clindamycin-induced *Clostridium difficile* colitis in hamsters receiving 25 mg/kg/day phenelfamycin A or vancomycin orally.

Four animals were used in each treatment group.
□ Control, △ phenelfamycin, ○ vancomycin.

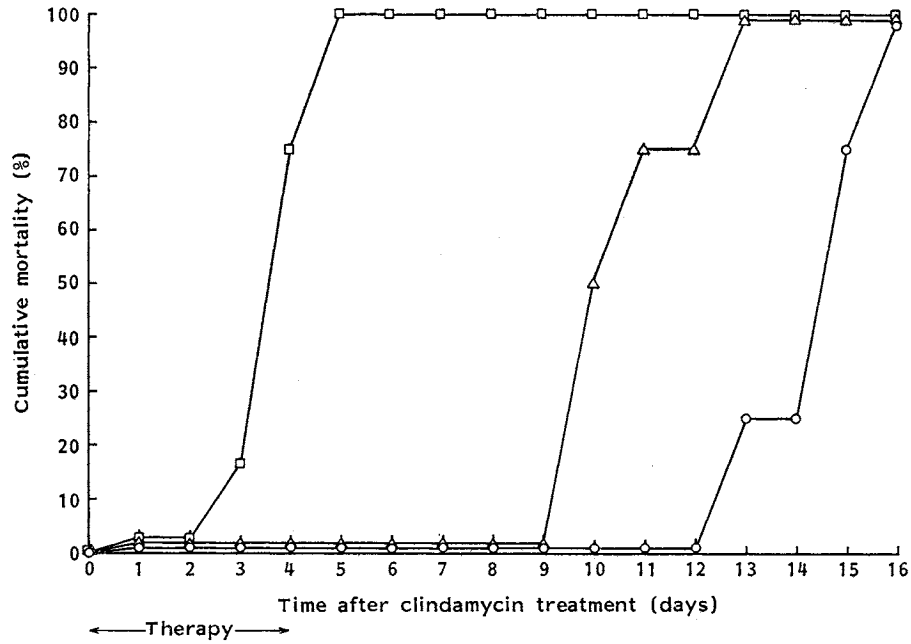

Table 5. Results of *Clostridium difficile* antigen tests on caecal samples from hamsters sacrificed 1, 4 and 7 days after clindamycin treatment.

Therapy[a]	Results of caecal contents taken on:[b]		
	Day 1	Day 4	Day 7
None	−−	++	++
Vancomycin (25 mg/kg/day)	−−	−−	−−
Phenelfamycin A (25 mg/kg/day)	−−	−−	−−

[a] Antibiotics were administered orally once a day for 5 days.
[b] Groups of two hamsters used. Results of *C. difficile* antigen test indicated by − for negative and + for positive.

therapy was 14.75, 11.0 and 4.0, respectively. Neither antibiotic prevented the eventual development of fatal colitis.

Results of antigen testing indicated that administration of vancomycin or phenelfamycin A delayed the appearance of *C. difficile* antigens (Table 5). Hamsters receiving treatment with either agent remained free of *C. difficile* antigens for at least 7 days after clindamycin challenge. Control animals, however, were positive for antigen as early as 3~4 days after clindamycin treatment. Positive caecal samples were removed from a control animal dying 3 days after clindamycin treatment and from 2 out of 2 hamsters sacrificed 4 days after clindamycin challenge.

Pharmacokinetic Studies

No phenelfamycin A was detected by HPLC analysis in the serum or urine of hamsters receiving

a single oral dose of 25 mg/kg. Levels of phenelfamycin A found in the caecal contents of these animals were 0.4, 0.8, 1.0 and 0.4 μg/g at 1, 2, 3 and 6 hours after administration, respectively.

Discussion

The phenelfamycins, composed of phenelfamycins A, B, C, E, F and unphenelfamycin, are new members of the elfamycin family of antibiotics that are produced by a *Streptomyces* sp.[5] *In vitro*, the phenelfamycins have activity against Gram-positive anaerobes, including *C. difficile*. In terms of their structure-activity against *C. difficile*, the trisaccharides, phenelfamycins E and F, are more potent than the disaccharide, phenelfamycin C, which is more potent than the monosaccharides, phenelfamycins A and B. The MICs of phenelfamycins E and F for *C. difficile* are generally within one to two 2-fold dilutions of that of efrotomycin.[14] Phenelfamycin A also has activity against Streptococci and *N. gonorrhoeae*. Unphenelfamycin is bacteriostatic for *C. difficile* ATCC 9689; the frequency of spontaneous resistance development by this organism to unphenelfamycin is low.

One of the phenelfamycins, phenelfamycin A, was tested for *in vivo* activity in a hamster model of *C. difficile* colitis. This compound was selected because of its *in vitro* potency and because it was available in sufficient quantities at the time of testing. Phenelfamycin A was also a candidate for *in vivo* testing because it was not adsorbed after oral administration in hamsters, a characteristic making this compound more desirable as a potential therapeutic agent against *C. difficile* colitis. When administered orally to hamsters phenelfamycin A delayed the development of *C. difficile* enterocolitis until after phenelfamycin therapy had ceased. Vancomycin, one of the antibiotics currently used clinically to treat *C. difficile* colitis, also delayed the onset of fatal colitis in hamsters, but did so for a slightly longer period of time. In the hamster colitis model, vancomycin prevents colonization with *C. difficile* while it is being administered, but may fail to correct the predisposing conditions within the gut that allow this bacterium to later cause disease.[15] Thus, the hamsters remain well until vancomycin therapy is discontinued, after which they develop a fatal enterocolitis because the gastrointestinal flora is not yet refractory to colonization with toxigenic *C. difficile*. From the present study it appears that the same situation is true in hamsters treated with phenelfamycin A.

It should be noted that phenelfamycin A and vancomycin are equally active against *C. difficile* ATCC 9689 *in vitro*. Other members of the phenelfamycin group, phenelfamycins E and F in particular, are more potent than vancomycin against this and other strains of *C. difficile in vitro*. Thus, other phenelfamycins may prove to be more effective in treating experimental *C. difficile* colitis than phenelfamycin A. Phenelfamycins E and F have not yet been tested in the hamster colitis model. Efrotomycin, which is more potent than both vancomycin or phenelfamycin A *in vitro* against *C. difficile*,[14] has been shown to protect hamsters from *C. difficile* colitis at a dose of 26 mg/kg.[16] Work with unphenelfamycin in the present study suggests that the potency of phenelfamycins may be altered in the presence of caecal contents. This confirms the necessity of testing these compounds in an animal model for antibiotic associated colitis.

The phenelfamycins are a novel family of elfamycins having several structural features not previously encountered in this class. None-the-less their biological properties appear to be very similar to those of other elfamycins.

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