QUATERNARY HETEROCYCLYLAMINO β-LACTAMS

V. L-640,876 TREATMENT OF INDUCED ENTEROTOXIGENIC COLIBACILLOSIS (SCOURS) IN CALVES AND PIGLETS

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A new semisynthetic cephalosporin antibiotic designated L-640,876, 7-β-(1-benzylpyridinium-4-yl)amino-3-{[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl}ceph-3-em-4-carboxylate, was highly active *in vitro* against 110 enteropathogenic strains of *Escherichia coli* and *Salmonella* species of animal origin. The MIC_{90} was 0.125 μg/ml for the *E. coli* strains, 2 μg/ml for the *S. choleraesuis* strains and 4 μg/ml for the *S. typhimurium* strains. In colostrum-fed calves infected with *E. coli* strain B44, L-640,876 administered by gavage at 30 mg/calf (0.67 mg/kg) twice a day for 3 days, starting at 20-hour post-inoculation, eliminated the diarrhea and reduced the mortality from 82% in the infected, nonmedicated calves to 11% in the infected, medicated calves ($P<0.05$). In colostrum-fed piglets infected with *E. coli* strain P155, L-640,876 administered by gavage at 12.5 or 20 mg/piglet (10 or 16 mg/kg) twice a day for 3 days, starting at 6-hour post-inoculation, eliminated the diarrhea and reduced the mortality from 79% in the infected, nonmedicated to 25% in the infected, medicated piglets ($P<0.05$). Thus, L-640,876 was highly effective in restoring the calves and piglets to good health by eliminating diarrhea and reducing mortality.

Enterotoxigenic *Escherichia coli* strains (ETEC) cause fatal diarrhea in newborn calves[1~5] and piglets[3,6,7]. These ETEC colonize the mucosal surface of the small intestine without tissue invasion, multiply to large numbers, and elaborate an enterotoxin(s) which causes the diarrhea.

In this paper, the antibacterial activity *in vitro* of L-640,876[8] against 110 enteropathogenic strains of *E. coli* and *Salmonella* species of animal origin is reported, and the efficacy of L-640,876 for treating induced enteric colibacillosis in dairy calves and piglets is described.

Materials and Methods

In Vitro Susceptibility Tests

Aqueous solutions of the following antibacterials were prepared just before use: cephamycin C (Merck), L-640,876 (Merck), apramycin (Lilly), spectinomycin (Upjohn) and tetracycline (Lederle). Serial two-fold dilutions of each compound were prepared in Müeller-Hinton agar.

Fifty-five strains of *E. coli* and 56 strains of *Salmonella* (*S. typhimurium* and *S. choleraesuis*) were used. Previously isolated from animal infections, these organisms were obtained from the National Animal Disease Laboratory (Ames, Iowa).

Cultures grown overnight in Trypticase soy broth were diluted such that when using a multipoint replicating device, a final inoculum size of 10^5 CFU/spot was achieved. The plates thus inoculated were incubated at 37°C for 18~24 hours before they were scored. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the test agent which inhibited visible growth of the organism on solid medium.

Challenge Cultures

A nalidixic acid resistant mutant of bovine ETEC B44 (09: K30: K99: H-), which produces only the heat-stable (ST) enterotoxin, was used in calves[1,3~5]. A nalidixic acid resistant mutant of porcine ETEC P155 (0149: K91: K88), which produces both the heat labile (LT) and heat-stable (ST) enterotoxins, was used in piglets[6]. Both strains produce a methanol-soluble ST which is active in infant mice[9]. The MIC of L-640,876 for each strain was 0.125 μg/ml.

Challenge Infection

All studies were conducted at Merck's Research Farm in Branchburg Township, N. J. Holstein or Holstein-Angus cross calves less than 15 hours old were acquired from local diary farms. The calves were placed in individual metal cages, 4 cages to each isolation room. Each calf received 1 liter of colostrum at the first feeding and 2 liters of whole milk twice daily thereafter. Calves were callenged orally with approximately 10^{11} colony-forming units (CFU) of strain B44 (washed cells in 50 ml saline) in the late afternoon of the day they arrived and always after the first feeding of colostrum. Unchallenged control calves were maintained in separate rooms. The fecal consistency of each calf was scored and a fecal sample or rectal swab was collected for culture each morning. All calves were necropsied the day they died or at the end of the experiment (10 days post-inoculation). Calves were weighed when received (weight range was 31 to 55 kg with a mean weight of 41 kg) and at necropsy.

Yorkshire pigs (Willow Grove Farm, Stroudsburg, Pa.) were used. The piglets were farrowed and kept with the sow in an isolation room throughout the experiment. Twelve to 18 hours after farrowing each piglet in a litter was challenged orally with approximately 10^{10} CFU of P155. Six-hour post-inoculation (PI), each litter was divided by sex and size into 2 equal groups. Each piglet in one group was medicated, while none of the piglets in the other group were medicated. The piglets were observed each morning when the fecal consistency of each piglet was scored and a fecal sample or swab was taken for culture. All piglets were necropsied the day they died or at the end of the experiment ($8 \sim 10$ days PI). At the time of inoculation, the weight range was 0.70 to 1.90 kg with a mean weight of 1.25 kg.

Clinical Observations and Bacteriology

Fecal consistencies of the calves and piglets were scored as follows: 3=profuse watery feces with little solid material; 2=watery feces with some solid material; 1=semisolid feces; 0=solid feces.

Fecal samples from calves were processed immediately after collection. B44 counts were made on homogenized fecal samples by serially diluting 1 g of wet feces with phosphate-saline solution. Appropriate dilutions were plated on Levine's EMB agar plates containing 100 μg/ml of nalidixic acid. The plates were incubated for 24 hours at 37°C and then *E. coli* colonies enumerated. Fecal swabs from piglets were qualitatively cultured for the presence of P155 by streaking them directly onto plates of modified Drigalski medium[10] containing 100 μg/ml of nalidixic acid. Blood cultures from representative live diarrheic calves or piglets were qualitatively cultured for strain B44 or P155. At necropsy, the heart, lungs, liver and spleen of each calf or piglet were qualitatively cultured for strain B44 or P155. Contents of the jejunum, ileum and colon of each calf were quantitatively cultured for strain B44, while in piglets, contents of these organs were qualitatively cultured for strain P155. Jejunal, ileal and colonic contents from several representative diarrheic piglets were quantitatively cultured for strain P155 by dilution in phosphate-saline and enumeration on modified Drigalski medium.

Medication

L-640,876 was given by gavage twice a day at 9: 00 a.m. and 4: 00 p.m. Immediately before dosing, the required amount of drug was dissolved in sterile H_2O; calves were dosed with a 10 ml volume and piglets with a 2 ml volume. In calves, medication commenced at 20-hour post-inoculation, in piglets, at 6-hour post-inoculation. Medication continued b.i.d. for 3 days.

Enterotoxin Assays

Sterile broth filtrates of ETEC B44 or P155 and small intestinal and fecal samples obtained at necropsy from representative infected, nonmedicated calves and piglets were individually assayed for ST activity in infant mice[11] and LT activity in an ELISA assay[12].

Results

In Vitro Susceptibility Tests

From the MIC_{90} data (Table 1), it is apparent that L-640,876 was the most active and the following relationship can be established in regard to the activities of these antibacterials: L-640,876>cephamy-

Table 1. *In vitro* susceptibility of enteropathogenic *E. coli* and *Salmonella* strains of animal origin to L-640,876 and other antibacterial agents.

Organism (No. of isolates)	Antibiotic	MIC (μg/ml)[a]		
		Range	For 50% of isolates	For 90% of isolates
E. coli (55)	Apramycin	2~64	8	16
	Cephamycin C	2~16	8	16
	L-640,876	0.008~0.25	0.06	0.125
	Spectinomycin	8~>128	16	>128
	Tetracycline	1~>128	>128	>128
S. typhimurium (28)	Apramycin	2~32	4	16
	Cephamycin C	2~8	4	8
	L-640,876	0.06~16	0.25	4
	Spectinomycin	16~>128	32	>128
	Tetracycline	4~>128	>128	>128
S. choleraesuis (27)	Apramycin	0.5~32	8	16
	Cephamycin C	2~8	4	4
	L-640,876	0.004~4	0.25	2
	Spectinomycin	16~>128	16	64
	Tetracycline	1~>128	2	>128

[a] Determined on Müeller-Hinton agar at 10^5 CFU/spot.

Table 2. L-640,876 treatment (orally at 30 mg per calf [0.67 mg/kg] b.i.d. for 3 days) of *E. coli* enteritis in calves[a].

Calves (No.)	Mortality[b] [deaths/ total (%)]	Mean diarrhea score[c] at day PI				Mean[d] B44 CFU/g feces at day PI				Mean weight loss [kg(%)]
		1	2	4	10	1	2	4	10	
Infected nonmedicated (11)	9/11 (82)*	3.0*	2.5*	—	—	2.7×10^{8}*	6.0×10^{5}*	—	—	5.0 (13)
Infected medicated (9)	1/ 9 (11)**	3.0*	1.3*	0.1	0.6	4.3×10^{5}**	9.8×10^{4}**	2.2×10^{1}	1.1×10^{3}	3.4* (8)
Noninfected nonmedicated (11)	2/11 (18)**	0.4**	1.2**	0.6	0.2	0	0	0	0	2.5* (6)

[a] Means for each variable with different numbers of asterisks are statistically significantly different ($P<0.01$). Fisher's exact test was used to analyze the mortality data; diarrhea scores, bacterial counts, and weights were analyzed by analysis of variance and Duncan's new multiple range test[13]. —, Not applicable.

[b] All deaths in the infected nonmedicated calves were due to the induced enteritis; 7 deaths occurred on day 2 PI, one on day 3 PI and one on day 5 PI. In infected, medicated calves, the one death was due to the induced enteritis whereas in noninfected, nonmedicated calves, the two deaths were due to nonspecific enteritis.

[c] 3, Profuse watery feces with little solid material; 2, watery feces with some solid material; 1, semisolid feces; 0, solid feces.

[d] Geometric mean.

cin C>apramycin>spectinomycin>tetracycline. Except for L-640,876 there was little difference between the *E. coli* and *Salmonella* strains in their susceptibility to these antibacterials. L-640,876 was at least 10× more active against the *E. coli* strains than against the *Salmonella* strains tested.

Calves

The disease syndrome which developed by 20-hour post-inoculation (PI) was characterized by profuse watery diarrhea, severe dehydration, anorexia, prostration and death generally at 2 days PI in nonmedicated calves. The jejunum, ileum and colon of these calves each contained $>10^9$ CFU of strain B44/g of contents. Samples from these three areas of the gut taken from representative calves produced gut weight to body weight ratios of >0.100 in infant mice which indicated the presence of ST throughout the gut[11].

L-640,876 reduced the mortality and eliminated the diarrhea and other symptoms (Table 2). Mortality was significantly reduced from 82% in the infected, nonmedicated controls to 11% in the infected, medicated calves. Medicated calves had a lower ($0.05<P<0.1$) diarrhea score and shed significantly fewer CFU of B44 in their feces after the first day of treatment (day 2 PI) with L-640,876. Mortality, diarrhea scores and weight loss were comparable for infected, medicated calves and noninfected, nonmedicated control calves over the 10-day experimental period.

Piglets

The disease syndrome which developed by 6-hour PI was characterized by profuse watery diarrhea,

Table 3. L-640,876 treatment [orally at 12.5 mg or 20 mg per piglet (10 or 16 mg/kg) b.i.d. for 3 days] of *E. coli* enteritis in suckling piglets[a].

Litter No.	Mortality data							
	Nonmedicated piglets				Medicated piglets			
	Alive	Dead	Total	% Mortality	Alive	Dead	Total	% Mortality
1	0	5	5	100	2	1	3	33
2	1	3	4	75	3	1	4	25
3	1	3	4	75	3	1	4	25
4	1	3	4	75	2	1	3	33
5	0	3	3	100	2	1	3	33
6	2	2	4	50	3	0	3	0
				79				25[b]

Medication	Mean diarrhea scores[c]			
	0 (6 hours)[d]	Days post-inoculation		
		1	2	3
None	2.5*	2.5*	1.3	—
L-640,876	2.2*	1.1**	0.1	0.0

[a] Litters 1~3 were treated with 12.5 mg/piglet and litters 4~6 were treated with 20 mg/piglet. First treatment was administered at 6-hour post-inoculation.

[b] Significant ($P<0.05$) reduction in mortality based on differences in mortality within litters using a paired t test.

[c] 3, Profuse watery feces with little solid material; 2, watery feces with some solid material; 1, semisolid feces; 0, solid feces.

[d] Means for each variable with different numbers of asterisks are statistically significantly different ($P<0.05$); paired t test based on differences between litters.

severe dehydration, anorexia, prostration, and death generally at 1 day PI in nonmedicated piglets. Thus, the disease syndrome was very similar in the calves and piglets. One noticeable difference was that the diarrhea appeared earlier after challenge in the piglets, and the nonmedicated piglets died earlier than the nonmedicated calves. On necropsy the entire gut of diarrheic piglets contained $>10^8$ CFU of strain P155 per g of gut contents. Jejunal, ileal, and colonic contents of representative diarrheic piglets produced gut weight to body weight ratios of >0.100 in infant mice, and were positive in the ELISA assay, which indicated the presence of ST and LT throughout the gut.

L-640,876 significantly ($P<0.05$) reduced the mortality from 79 to 25% and eliminated the diarrhea in surviving piglets. The two dosage levels were equally effective and, therefore, these data were combined. A significant reduction in the mean diarrhea score of medicated piglets was observed on day 1 PI, and these piglets had solid feces by day 2 PI. None of the medicated piglets were shedding the challenge P155 *E. coli* in their feces after day 5 PI. The final mean weight of medicated piglets was 2.24 kg with an average gain of 0.91 kg per piglet. In contrast, the final mean weight of the nonmedicated piglets was 1.15 kg with an average loss of 0.03 kg per piglet.

Discussion

The disease syndromes in both calves and piglets were similar to those described by other investigators[1,2,4~7] and by us in our studies with cephamycin C[3]. L-640,876, like cephamycin C, was highly effective in restoring the calves and piglets to good health by eliminating diarrhea and reducing mortality. L-640,876 in calves and piglets reduced the mortality from 82 to 11% and 79 to 25%, respectively, while cephamycin C produced similar reductions of 90 to 14% and 75 to 31%. These data, combined with the *in vitro* activity against *Salmonella* species, indicate that L-640,876 should be a useful drug for treatment of gastrointestinal diseases caused by *E. coli* and *Salmonella* species in livestock.

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