QUATERNARY HETEROCYCLYLAMINO β-LACTAMS

IV. COMPARISON OF THE *IN VIVO* ANTIBACTERIAL ACTIVITIES OF L-640,876, MECILLINAM, CEFOXITIN AND CEFOTAXIME

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The novel β-lactam, L-640,876, exhibited excellent therapeutic activity when administered parenterally but not orally to mice infected with a variety of pathogenic bacteria. In this respect, the compound was as potent as cefotaxime against representative Gram-positive and Gram-negative organisms, in most cases, equal to or more potent than cefoxitin, and more effective than mecillinam. When administered subcutaneously to normal mice at dose levels ranging from 10 to 50 mg/kg, L-640,876 provided an adequate dose response, recovery of *ca.* 45% of biological activity in the urine, and excellent distribution at the highest dose level into liver, lung, kidney, heart muscle, but not brain.

The semisynthetic cephalosporin, L-640,876, is a member of a new class of quaternary heterocyclylamino β-lactam antibiotics recently described by chemists at the Merck Sharp & Dohme Research Laboratories[1]. It has been shown to have a potent, broad spectrum of activity *in vitro*[2].

We compared the effectiveness of L-640,876 with that of mecillinam, cefoxitin and cefotaxime against experimental septicemia in mice and investigated the influence of inoculum size and β-lactamase production of the infecting organism on the outcome of chemotherapy.

Materials and Methods

Antibiotics

Laboratory powders of L-640,876 and cefoxitin sodium (Merck & Co., Inc.), cefotaxime (Hoechst AG, Frankfurt) and mecillinam (Leo Pharmaceutical Products, Ballerup) were used in these studies. All possessed a potency of greater than 900 μg/mg.

Animals

Female CD1 mice were purchased from Charles River Breeding Laboratories, Wilmington, MA. They were housed in a temperature-controlled environment and fed Purina Formulab Chow # 5008 and provided tap water *ad libitum*.

Efficacy Studies

Mice weighing, on the average, 19~22 g were injected intraperitoneally (i.p.) with 0.5 ml of an appropriate dilution of the pathogen in brain heart infusion broth (Difco) (for *Streptococcus pyogenes* MB 2874 and *Klebsiella pneumoniae* MB 4005) or in 5% hog gastric mucin (Wilson, Division of Inolex Corp., Park Forest South, IL). All infected, untreated mice died within 48 hours.

Drugs were given as aqueous solutions by the subcutaneous (s.c.) route in divided doses at 0 and 6 hours after infection. Each therapy group consisted of at least five mice; four fourfold dilutions of each antibiotic were tested. All animals were observed for 7 days after infection and the number of survivors on that day was used to calculate the median effective dose (ED_{50}) by the method of KNUDSEN and CURTIS[3].

Pharmacokinetics

Groups of five 20 g mice were given a single s.c. dose of 10, 20 or 50 mg/kg of L-640,876 and 0.5 ml water by gavage to stimulate urine flow. The five mice were then placed in a metabolism cage designed to collect urine free from fecal contamination. At a specified time, a group was bled by heart puncture, the pooled blood was allowed to clot and the serum was removed after centrifugation. At the same time, urine from that group was collected and the volume measured. All serum and urine samples were stored at −20°C until bioassayed.

In other groups of mice given 50 mg/kg of L-640,876 s.c., livers, left kidneys, lungs, hearts and brains in addition to blood, were removed at 1, 2 and 4 hours after dosing. The tissues were homogenized in SORENSEN's buffer (pH 7) and adjusted to 200 mg tissue/ml. These samples also were stored at −20°C until bioassayed.

Agar-Dilution Susceptibility Tests

The minimum inhibitory concentration (MIC) was determined in an agar-dilution assay using nutrient agar or nutrient agar supplemented with 10% horse serum (for *Streptococcus pyogenes* MB 2874). Overnight cultures and appropriate dilutions thereof were deposited with the aid of an automated multipronged inoculator onto the surface of the agar which contained doubling-dilutions of antibiotic. The final inoculum for each of the organisms used later in the mouse protection tests was 10^4 CFU/spot. For the 3 organisms used in studies of the *in vivo* inoculum effect, the CFU/spot corresponded to the CFU in the challenge dose. The test plates were incubated at 35°C for 18 hours. The MIC was defined as the lowest concentration of antibiotic that suppressed visible growth.

Microbiological Assays

The antibiotic content of serum, urine and tissues was determined by standard disc diffusion procedures using nutrient agar supplemented with 0.2% yeast extract. *Escherichia coli* MB 2891 (for serum and urine) and *E. coli* MB 4269 (for tissues) served as the assay organisms. Serum samples were diluted in normal mouse serum, urine in SORENSEN's buffer (pH 6) and tissues in SORENSEN's buffer (pH 7). Potencies were calculated from the appropriate standard curves, in some cases with the aid of a computer program.

Cultures

All bacterial cultures were of human or animal origin and were selected because of their virulence for mice. They were maintained in the lyophilized state before use. Identifying numbers are those of the Merck stock culture collection.

Results and Discussion

Chemotherapeutic Trials

The therapeutic effectiveness of L-640,876 was demonstrated against experimental systemic infections in mice. Results of these studies in comparison with mecillinam and cefotaxime are shown in Table 1. The ED_{50} values are from single trials in which all three antibiotics were tested simultaneously. They are representative of values obtained from other mouse protection tests in which only 1 or 2 of the antibiotics was evaluated.

The potent, broad spectrum activity of L-640,876 seen *in vitro*[2] was also evident *in vivo* when the antibiotic was given by the s.c. route to mice infected with any of a number of Gram-positive or Gram-negative pathogens. In general, the MIC was a good predictor of *in vivo* efficacy.

L-640,876 was more effective than mecillinam and comparable to cefotaxime in protecting mice against infections with staphylococci and streptococci.

Against the Gram-negative pathogens, L-640,876 was as effective as mecillinam against *Enterobacter cloacae, Salmonella schottmuelleri* and the 3 strains of *E. coli* tested. It was considerably more active than mecillinam against *Proteus morganii, Proteus mirabilis, Proteus vulgaris* and *Klebsiella*

Table 1. Comparison of the therapeutic efficacy of L-640,876, mecillinam and cefotaxime in experimental bacteremia in mice.

Pathogen	MB No.	β-Lactamase	Challenge dose[a]		L-640,876		Mecillinam		Cefotaxime	
			CFU	LD_{50}	MIC[b] (μg/ml)	ED_{50}[c] (mg/kg)	MIC (μg/ml)	ED_{50} (mg/kg)	MIC (μg/ml)	ED_{50} (mg/kg)
Staphylococcus aureus	2865	+	8.70×10^2	2890	0.25	1.24	16	7.85	0.5	0.83
Streptococcus pyogenes	2874	−	6.85×10^2	317	<0.02	0.24	2	29.00	<0.02	0.06
Enterobacter cloacae	2646	+	1.60×10^7	513	0.25	10.75	0.06	15.65	128	200
Escherichia coli	2884	+	8.40×10^2	750	<0.02	0.06	0.03	0.08	0.25	0.06
E. coli	2891	+	5.30×10^1	73	<0.02	0.04	0.03	0.13	16	3.78
E. coli	4363	+	6.08×10^5	520	0.06	0.25	0.06	0.63	0.13	0.06
Klebsiella pneumoniae	4005	−	3.20×10^4	299	<0.02	30.20	0.06	>200	<0.02	2.05
Proteus morganii	2833	+	3.93×10^5	298	0.06	0.39	16	44.40	2	0.83
P. mirabilis	3125	−	4.00×10^4	290	0.03	0.94	0.25	56.00	<0.02	0.01
P. vulgaris	2829	+	4.53×10^5	>1,000	<0.02	0.09	0.06	7.25	<0.02	0.26
Salmonella schottmuelleri	2837	−	3.57×10^2	110	<0.02	0.05	0.02	0.08	0.03	0.02

[a] Infection was established by the intraperitoneal injection of an appropriate dilution of the pathogen in brain heart infusion broth (*Streptococcus pyogenes, Klebsiella pneumoniae*) or in 5% hog gastric mucin; colony-forming units (CFU) were calculated using standard plate counting techniques and the number of median lethal doses (LD_{50}) by the method of KNUDSEN and CURTIS[5].

[b] Minimum inhibitory concentration (MIC) in μg/ml at 10^4 CFU applied to the agar surface with a multipoint inoculator.

[c] Median effective dose (ED_{50}) in mg/kg/dose; drugs were administered by the subcutaneous route in divided doses at 0 and 6 hours after infection.

Table 2. Antibacterial activity of L-640,876 *in vitro* and *in vivo* as a function of inoculum size/challenge dose.

Pathogen		CFU in challenge dose	L-640,876		Mecillinam		Cefotaxime		Cefoxitin	
			MIC[a]	ED_{50}[b]	MIC	ED_{50}	MIC	ED_{50}	MIC	ED_{50}
Escherichia coli	MB 2891[d]	10^2	<0.016	0.06	<0.016	0.03	4	6.25	64	100
		10^4	<0.016	0.21	0.031	1.35	16	15.1	128	60.5
		10^6	0.031	4.08	0.063	20.0	32	184	128	267.5
Proteus morganii	MB 2833[d]	10^5	0.063	0.39	16	44.4	2	0.83	8	2.31
		10^7	8.0	47.1	>128	NT[c]	8	13.25	32	6.25
Proteus mirabilis	MB 3125	10^4	0.031	0.53	0.25	29.0	<0.016	0.01	2	6.60
		10^6	1.0	20.65	64	>400	0.031	0.07	4	20.65

[a] MIC in μg/ml; determined in an agar-dilution assay using nutrient agar and a multipronged inoculator.

[b] ED_{50} in mg/kg; antibiotics were given s.c. at 0 and 6 hours after infection.

[c] NT=not tested.

[d] β-Lactamase producing strain.

pneumoniae. This latter infection was refractory to treatment with mecillinam although the organism was susceptible to the antibiotic *in vitro*.

In general, L-640,876 was as efficacious as cefotaxime in protecting mice against most infections caused by Gram-negative organisms. There were two notable exceptions. The *Enterobacter* infection was controlled by L-640,876 at lower doses than those required for cefotaxime, as might be expected from the respective MICs. On the other hand, *Klebsiella pneumoniae*, which was equally susceptible to both antibiotics *in vitro*, was less well controlled by L-640,876 than by cefotaxime (15-fold more active) *in vivo*. The *Klebsiella* and *Enterobacter* were the only organisms for which the excellent *in vitro* activity of L-640,876 was not translated into highly effective therapy *in vivo* in single digit or lower values.

In Vivo Inoculum Effect

KOUPAL *et al.*[2] showed that increasing the inoculum size of certain Gram-negative bacteria increased the MIC of L-640,876 and that this increase was associated with stability of the cephem to the β-lactamase elaborated by the organism or the presence of certain salts in the culture medium. To determine whether the ED_{50} would also be influenced by increases in inoculum size, mouse protection tests were performed in which the challenge doses were increased in 100-fold increments. Antibiotics were administered by s.c. injection immediately after infection and again 6 hours later. The pathogens studied were *E. coli* MB 2891 and *Proteus morganii* MB 2833, known β-lactamase producers, and *Proteus mirabilis* MB 3125, a strain that does not produce β-lactamase. Results of these tests are shown in Table 2.

In the study with *E. coli* MB 2891, a strain that was highly susceptible to L-640,876 *in vitro*, a ten thousand-fold increase in the challenge dose increased the ED_{50} only 68-fold from 0.06 mg/kg to 4.08 mg/kg. The increase in inoculum size had little effect on the MIC of mecillinam but raised the ED_{50} more than 600-fold from 0.03 mg/kg to 20 mg/kg. In contrast, the MICs and ED_{50}s for cefotaxime were adversely affected by increasing inoculum for this strain of *E. coli*. Although this organism is considered to be resistant to cefoxitin, increases in inoculum size had little or no effect on the MIC or ED_{50} of this antibiotic.

In studies with *Proteus morganii* MB 2833 and *Proteus mirabilis* MB 3125, large increases in MICs of L-640,876 were seen with 100-fold increases in inoculum size, and significantly greater amounts of the antibiotic were required to protect mice against bacteremia induced by both of these pathogens as the challenge dose was increased. Mecillinam was considerably less active against the smaller inoculum than was L-640,876 and ineffective when tested against the larger inoculum both *in vitro* and *in vivo*. The MICs and ED_{50}s of cefotaxime and cefoxitin were only slightly affected by increases in inoculum sizes of these pathogens.

Since significant shifts in MIC as a function of inoculum size for both L-640,876 and mecillinam are known to be due to β-lactamase production, conductivity effected by certain salts including sodium chloride, or both[2], the reason(s) for the higher dose required to protect infected animals under these conditions must be approached with caution. Suffice it to say that, in the case of the *Proteus* strain, the MIC is a good predictor of ED_{50}. The lack of correlation of MIC and ED_{50} for the *E. coli* strain, especially in view of the high resistance of L-640,876 to the β-lactamase produced by the organism, at present cannot be explained.

Pharmacokinetics

In groups of mice given a single s.c. dose of 10, 20 or 50 mg/kg of L-640,876, peak serum concentra-

Table 3. Antibiotic concentrations in serum and urine of mice given a single subcutaneous dose of L-640,876: dose response.

Dose (mg/kg)	Concentration (μg/ml) in serum at hour					$t_{\frac{1}{2}}$[a] (minutes)	AUC[a] (μg-hours/ml)	Urinary recovery in 4 hours	
	0.25	0.5	1.0	2.0	4.0			(μg)	(%)
50	72.5	32.0	15.0	4.8	<0.5[b]	36	45.8	2,090	41.8
20	17.8	15.0	7.4	1.6	<0.5	27	16.5	920	46.0
10	12.4	10.5	4.6	0.5	<0.5	19	10.1	487	48.7

[a] $t_{\frac{1}{2}}$=Serum half-life, estimated from the terminal linear portion of the serum concentration *vs.* time curve; AUC=Area under the serum concentration *vs.* time curve. A computer program was used to calculate these parameters.

[b] Sensitivity of assay=0.5 μg/ml; assay organism was *E. coli* MB 2891.

tions, half-lifes ($t_{\frac{1}{2}}$) and areas under the serum concentration *vs.* time curves (AUC) increased as the dose increased (Table 3). Serum peaks were observed at 15 minutes after each dose and ranged from a high of 72.5 μg/ml to a low of 12.4 μg/ml. The $t_{\frac{1}{2}}$ values were 36, 27 and 19 minutes and AUC, 45.8, 16.5 and 10.1 μg-hours/ml following the 50, 20 and 10 mg/kg doses, respectively.

Concentrations of antibiotic in all urine samples exceeded 100 μg/ml at 1, 2 and 4 hours after dosing and total recovery of antibiotic activity increased as the dose increased. The cumulative percent of dose excreted in urine during the four hour interval after dosing varied inversely with the dose but remained fairly constant over the range tested, 41.8% after the 50 mg/kg dose to 48.7% after the 10 mg/kg dose. The antibiotic was not absorbed when given to mice by gavage.

Table 4. Antibiotic concentration in tissues of mice given a dose of 50 mg of L-640,876 per kg of animal body weight by the subcutaneous route.

Tissue	Antibiotic concentration (μg/ml or μg/g) at		
	1 hour	2 hours	4 hours
Serums	13.1	2.4	0.08
Liver	26.45	4.45	1.35
Kidney	10.85	0.75	<0.1
Lung	3.60	0.35	<0.1
Heart	1.70	0.1	<0.1
Brain	<0.1[a]	<0.1	<0.1

[a] Sensitivity of assay=0.02 μg/ml; assay organism was *E. coli* MB 4269. Potencies were determined with the aid of a computer-assisted image analyzer.

In other groups of mice given 50 mg/kg of L-640,876 s.c., the antibiotic content in liver, kidney, lung, heart and brain, in addition to serum, was determined at 1, 2 and 4 hours after dosing. The results are shown in Table 4.

Serum values were comparable to those obtained from mice used in the study reported in Table 3. Among the solid tissues, liver was found to contain the highest amount of antibiotic activity (Table 4), approximately twice that in serum at 1 and 2 hours after dosing. Antibiotic activity in the kidney approached that in the serum at 1 hour after the 50 mg/kg dose (10.85 μg/g compared to 13.0 μg/ml) but declined rapidly thereafter. Lung and heart contained 3.6 and 1.7 μg/g, respectively, at 1 hour after dosing and little or no activity at the other times sampled. No activity was detected in brain tissue (sensitivity of bioassay was 0.02 μg/ml) indicating no passage of the antibiotic across the normal meninges of young adult mice under the conditions of this experiment.

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