L-658,310, A NEW INJECTABLE CEPHALOSPORIN

III. EXPERIMENTAL CHEMOTHERAPEUTICS AND PHARMACOKINETICS IN LABORATORY ANIMALS†

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The therapeutic activity of L-658,310 was demonstrated in experimental bacteremias in normal, diabetic and neutropenic mice. Especially potent activity was shown against the usually difficult to control pathogens, *Enterobacter cloacae* and *Pseudomonas aeruginosa*, that were resistant to ceftazidime and/or gentamicin. Pharmacokinetic studies in mice showed a linear dose response in serum after the 20 and 50 mg/kg subcutaneous dose and urinary recoveries of administered dose of about 60% in 6 hours. Excretion was mainly by glomerular filtration. In a crossover design in rhesus monkeys, the pharmacokinetics of L-658,310 were similar to those of ceftazidime and suggest a moderately long half-life in serum of humans.

L-658,310 (BO-1236) is a new semisynthetic cephalosporin that has been shown to possess superior activity against *Pseudomonas aeruginosa* and other glucose non-fermenters both *in vitro* and *in vivo*[2,3].

The present report confirms the excellent chemotherapeutic activity of L-658,310 in normal mice and extends this observation to diabetic and neutropenic mice. In addition, data from pharmacokinetic studies in mice and in rhesus monkeys are presented.

Materials and Methods

Antibiotics

Laboratory powders of L-658,310 and norfloxacin, each greater than 99.5% pure, commercially available ceftazidime (Glaxo Group Research Ltd., Fortaz, ceftazidime for injection) and gentamicin (Schering Corporation, Garamycin, gentamicin sulfate) were used in these studies. L-658,310 was prepared in 0.07 M SORENSEN's phosphate buffer pH 7.2, and norfloxacin in 0.05 N NaOH. Ceftazidime and gentamicin were diluted in sterile distilled water.

Animals

Female CDl mice were purchased from Charles River Breeding Laboratories, Wilmington, MA. They were housed in a temperature- and humidity-controlled environment and were given Purina Formulab Chow No. 5008 and tap water *ad libitum*.

Three male rhesus monkeys, identified as 83-233, 83-234 and 83-235, were healthy young adults that were purchased from Charles River Breeding Laboratories, Key Lois, FL. When not on test, they were housed in individual cages in rooms maintained at 22±0.5°C and were offered Purina Monkey Chow No. 5037 and fruit (1/4 orange or 1/4 apple) twice daily. Water was available *ad libitum*.

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Experimental Chemotherapy in Normal Mice

Experimental systemic infections were established by ip injection of a suitable dilution of the pathogen in 5% hog gastric mucin (Wilson, Division of Inolex Corp., Park Forest South, IL) or in brain heart infusion (Difco) for *Klebsiella pneumoniae* MB 4005 and *Pseudomonas aeruginosa* MB 2835. Each challenge dose contained at least 25 LD_{50}s and all infected, untreated mice died within 48 hours. Treatment was by the sc route immediately after infection and again 6 hours later. When norfloxacin was used as a comparative agent, it was administered by gavage. At least four 4-fold dilutions of each agent were tested. Each therapy group consisted of five mice weighing, on average, 19~22 g. The mice 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[4].

Experimental Chemotherapy in Diabetic and Neutropenic Mice

Diabetes was induced by a single ip injection of a 200 mg/kg dose of streptozotocin (Calbiochem-Behring, San Diego, CA). One week later, mice with urinary glucose concentrations of 500 mg/dl or greater were selected for the mouse protection test.

Neutropenia was produced by two sc injections of cyclophosphamide (Bristol-Meyers Co., Cytoxan): The first dose, 200 mg/kg, 4 days before infection and the second dose, 100 mg/kg, on the day before infection. This treatment reduced the total white blood cell (WBC) count to about 1,000/mm^3 and the number of neutrophils to $<50/mm^3$ at the time of infection. Total WBC counts for normal mice were 9,000~10,000/mm^3, and examination of blood smears showed that about 25% of the WBC were polymorphonuclear neutrophils.

Infections in the diabetic and neutropenic mice were established as described above for normal mice.

Pharmacokinetics in Mice

Groups of five 20 g CDl female mice were given a single dose of 20 or 50 mg/kg of L-658,310 by the sc route and 0.5 ml water by gavage to stimulate urine flow. One group of five mice was placed in a metabolism cage designed to collect the urine free from fecal contamination. At a specified time after dosing, blood and urine samples were collected from a group. The pooled blood was allowed to clot and the serum was removed after centrifugation and stored at −20°C until assayed for total antibacterial activity. The urine volume was measured and the sample was frozen prior to bioassay. In addition, tissues were taken at 1, 2, 4 and 6 hours from mice given the 50 mg/kg dose. The soft tissues (kidneys, liver, lungs, thigh muscle and brain) were homogenized in SORENSEN's buffer pH 7 and adjusted to 200 mg tissue/ml. The femurs were frozen in a dry ice/acetone bath, crushed, and the antibiotic was eluted in two volumes (w/v) of buffer overnight at 5°C. These samples also were frozen until bioassayed.

To determine whether urinary excretion of L-658,310 is by glomerular filtration or tubular secretion, other groups of mice were given a 500 mg/kg dose of probenecid (Sigma Chemical Company, St. Louis, MO) by gavage immediately before the 20 mg/kg sc dose of L-658,310. Serum and urine samples were collected for bioassay as described above.

Pharmacokinetics in Rhesus Monkeys

On the day before a test, the monkeys were removed from the cages under ketamine (Parke-Davis, Vetalar, ketamine HCl injection, USP) anesthesia, 10 mg/kg im, weighed and placed in chairs designed to restrain the monkey with no discomfort.

The pharmacokinetics of L-658,310 was compared with ceftazidime in a crossover design. A 10 mg/kg dose of the antibiotic was administered into the *vastus lateralis* muscle. Blood samples were taken from the femoral vessels at intervals from 15 minutes to 6 hours after dosing. Urine was collected in a flask set in ice water for the periods 0~6 hours and 6~24 hours. Antibiotic activity in serum and urine was determined by bioassay.

Ceftazidime was tested first and L-658,310 7 weeks later. Average weight of the monkeys was 4.1 kg at the time of the ceftazidime study and 4.6 kg when L-658,310 was tested.

The area under the serum concentration/time curve extrapolated to infinity (AUC) and the serum

half-life estimated from the terminal linear portion of the serum concentration/time curve ($T_{1/2}$) were calculated with the aid of a computer program.

Binding to Human Plasma Proteins

Binding to human plasma proteins was determined as described in PELAK *et al.*[5]. Briefly, the plasma/antibiotic mixture was placed in the upper chamber of an Amicon Centricon-10 microconcentrator and centrifuged at 4,500×*g* for 30 minutes. The ultrafiltrate was analyzed in a Gilford UV/VIS "Response" scanning spectrophotometer.

Binding was calculated as follows:

$$\frac{\text{Peak absorption (B)} - \text{Peak absorption (U)}}{\text{Peak absorption (B)}} \times 100 = \% \text{ Bound},$$

where B=buffer and U=ultrafiltrate.

Peak absorption for L-658,310 was at 202.5 nm and for ceftazidime, 256.5 nm.

Microbiological Assays

Standard disk diffusion procedures were used to determine total bioactivity in assay specimens. The bioassay for L-658,310 employed either *Escherichia coli* MB 4353 or *Proteus mirabilis* MB 838 in nutrient agar (Difco) supplemented with 0.2% yeast extract. The procedure for ceftazidime employed *P. mirabilis* MB 838 in Antibiotic Medium No. 11 (Difco). Standard curves were prepared in normal serum of the test species for assay of serum samples and in 0.07 M SORENSEN's phosphate buffer pH 6.8 for assay of L-658,310 in urine and in 1% phosphate buffer pH 7.0 for assay of ceftazidime in urine.

After overnight incubation at 37°C, zones of inhibition were measured with a computer-assisted image analyzer, and potencies were calculated from the appropriate standard curve. Assay sensitivity was 0.1 μg/ml for L-658,310 and 0.3 μg/ml for ceftazidime.

Bacterial Cultures

The bacterial strains used in these studies were of human origin and were selected for virulence to mice (except for organisms used in bioassays). They were maintained in the lyophilized state and reconstituted in Brain Heart Infusion for use. The identifying numbers are those of the Merck Clinical Microbiology Services Laboratory (CL) or of the Merck Sharp & Dohme Research Laboratories stock culture collection (MB).

Results

Chemotherapeutic Trials

The therapeutic effectiveness of L-658,310 was demonstrated in a variety of experimental bacteremias in normal, diabetic and neutropenic mice. Results of studies in normal mice are presented in Table 1.

Although L-658,310 has poor activity against Staphylococci[2,3] *in vitro*, it did protect mice infected with *Staphylococcus aureus* MB 2865, a strain that is susceptible to most penicillins and cephalosporins. It was, however, less effective than either ceftazidime or gentamicin.

L-658,310 demonstrated excellent activity against bacteremias due to Gram-negative organisms. It was better than ceftazidime and as effective as gentamicin against the potent β-lactamase producers, *Enterobacter cloacae* MB 2646 and *E. coli* MB 2891. L-658,310 was equivalent to ceftazidime and gentamicin when used as therapy against *K. pneumoniae* MB 4005, *P. mirabilis* MB 2830 and *P. aeruginosa* MB 2835. It also cured mice infected with *P. aeruginosa* MB 4711 and *P. aeruginosa* CL 3092, strains that are resistant *in vitro* to ceftazidime and gentamicin. Although ED_{50} values for gentamicin against *P. aeruginosa* MB 4711 and *P. aeruginosa* CL 3092 are comparable to those of L-

Table 1. Therapeutic efficacy of L-658,310, ceftazidime and gentamicin in experimental systemic infections in normal mice[a].

Pathogen	Strain No.	L-658,310		Ceftazidime		Gentamicin	
		MIC[b]	ED_{50}[c]	MIC	ED_{50}	MIC	ED_{50}
Staphylococcus aureus	MB 2865	32.0	41.38	4.0	5.16	0.25	0.08
Enterobacter cloacae	MB 2646	4.0	1.56	128.0	50.0	1.0	0.18
Escherichia coli	MB 2891	4.0	0.24	64.0	5.88	8.0	1.5
Klebsiella pneumoniae	MB 4005	0.25	6.6	0.03	1.56	2.0	0.84
Proteus mirabilis	MB 2830	0.125	0.26	0.06	0.08	8.0	0.63
Pseudomonas aeruginosa	MB 2835	2.0	5.88	1.0	5.40	2.0	3.40
	MB 4711	2.0	33.7	32.0	170.9	8.0	25.0
	CL 3092	8.0	62.5	128.0	143.0	16.0	40.0

[a] Infection induced by ip injection of the pathogen in broth (MB 4005 and MB 2835) or 5% hog gastric mucin; the challenge doses contained at least $25 \times LD_{50}$.
[b] MIC in μg/ml; determined in agar dilution assay vs. 10^5 cfu/spot.
[c] Therapy was by the sc route at 0 and 6 hours after infection; ED_{50} in mg/kg/dose.

Table 2. Mean ED_{50} values for L-658,310, gentamicin and norfloxacin against *Enterobacter cloacae* and *Pseudomonas aeruginosa* bacteremias in mice.

Pathogen	Therapy	Route	Number of tests	ED_{50} (mg/kg/dose)[b] (95% confidence limits)
E. cloacae MB 2646	L-658,310	sc	3	1.75 (0.50~6.12)
	Gentamicin	sc	5	0.43 (0.18~0.99)
P. aeruginosa CL 3092	L-658,310	sc	4	50.4 (24.8~102)
	Gentamicin	sc	4	45.7 (32.1~65.0)
	Norfloxacin	po	6	53.8 (33.1~87.4)

[a] Therapy was administered at 0 and 6 hours after infection.
[b] Geometric mean ED_{50}.

658,310, the latter, like most other cephalosporins, is a safer antibacterial agent. The toxic dose 50% (TD_{50}) of gentamicin is 300 mg/kg × 2 sc doses in normal mice and may be lower in infected mice. Therefore, the therapeutic index (TD_{50}/ED_{50}) of gentamicin is $\leq$12 against *P. aeruginosa* MB 4711 and $\leq$7.4 against *P. aeruginosa* CL 3092. By comparison, the TD_{50} of L-658,310 is 2,201 mg/kg iv[2], and may be higher after sc administration. The therapeutic indices for L-658,310 are thus estimated to be $\geq$65 and $\geq$35 against *P. aeruginosa* MB 4711 and *P. aeruginosa* CL 3092, respectively.

The data presented in Table 1 are from single trials in which all three antibiotics were tested simultaneously and are representative of values obtained in other studies in which only one or two of the agents was tested. In Table 2, geometric mean ED_{50} values and 95% confidence limits are given for L-658,310, gentamicin and norfloxacin against the usually difficult to control pathogens, *E. cloacae* MB 2646 and *P. aeruginosa* CL 3092. On repeat trials, the consistently effective therapy provided by L-658,310 compared favorably with gentamicin and norfloxacin.

In diabetic mice infected with *P. aeruginosa* CL 2860, a clinical isolate resistant to ceftazidime, the ED_{50} of L-658,310 was only about twice that in normal mice (Table 3). Gentamicin had slightly lower ED_{50} values in both the normal and diabetic mice. Ceftazidime also afforded protection, but the doses required were considerably higher than those of L-658,310 or gentamicin.

In neutropenic mice infected with *P. aeruginosa* CL 2860 (Table 3), gentamicin was the most effective agent. Norfloxacin, administered by gavage, provided better protection than L-658,310 given

Table 3. Therapeutic efficacy of L-658,310 in experimental bacteremias due to *Pseudomonas aeruginosa* in normal, diabetic and neutropenic mice.

	Challenge dose[a]		ED_{50}[b]			
	cfu	LD_{50}	L-658,310	Ceftazidime	Gentamicin	Norfloxacin
P. aeruginosa CL 2860						
Normal	3.9×10^6	57	10.8	117	<6.3	NT
Diabetic	3.9×10^6	162	26.5	200	9.4	NT
Normal	6.9×10^8	403	28.2	113	13.3	41.4
Neutropenic	6.9×10^4	>10,000	94.0	341	23.6	62.5
P. aeruginosa CL 3092						
Normal	5.3×10^6	89	28.2	>400	32.7	50
Neutropenic	5.3×10^4	>1,000	100	>400	100	400

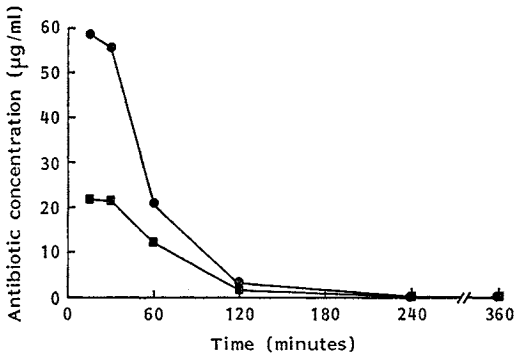
[a] Infection was induced by ip injection of the pathogen in 5% hog gastric mucin.
[b] Median effective dose in mg/kg/dose; therapy was administered at 0 and 6 hours after infection by the sc route for all agents except norfloxacin which was given by gavage.
NT: Not tested.

subcutaneously, but the difference was not significant. As expected, ceftazidime was the least effective agent in this trial.

When *P. aeruginosa* CL 3092, a clinical isolate resistant *in vitro* to both ceftazidime and gentamicin, was used as the infecting organism, the ED_{50} for L-658,310 was 28.2 mg/kg in normal mice and 100 mg/kg in neutropenic mice, values that were similar to those of gentamicin. Norfloxacin had slightly higher ED_{50}s in this single trial, but the values are not significantly different from those of L-658,310 and gentamicin. Ceftazidime did not protect mice at the highest doses tested; the ED_{50} was >400 mg/kg in both the normal and neutropenic mice.

In summary, L-658,310 has been shown to be efficacious in experimental bacteremias in normal, diabetic and neutropenic mice.

Fig. 1. Concentrations of L-658,310 in serum of mice following administration of a 50 mg/kg or 20 mg/kg dose by the sc route.



Dose	Cmax (μg/ml)	AUC (μg·hour/ml)	$T_{1/2}$ (minutes)
50 mg/kg (●)	58.8	52	31
20 mg/kg (■)	21.9	23	21

Pharmacokinetics in Mice

Pharmacokinetic studies in mice demonstrated the efficient absorption, distribution and elimination of L-658,310. Serum concentrations, presented graphically in Fig. 1, showed a linear dose response after the 50 and 20 mg/kg sc doses. The peak concentrations at 15 minutes after dosing were 58.8 and 21.9 μg/ml after the 50 and 20 mg/kg dose, respectively, and dropped to 1~3 μg/ml at 2 hours. AUC and $T_{1/2}$ were 52 μg·hour/ml and 31 minutes after the 50 mg/kg dose and 23 μg·hour/ml and 21 minutes after the 20 mg/kg dose. L-658,310 was excreted rapidly into the urine; concentrations exceeded 200 μg/ml in all samples. Urinary recovery of administered dose was about 60% in 6 hours.

The pharmacokinetics of L-658,310 were similar to those of ceftazidime. Serum parameters for

Table 4. Concentrations of L-658,310 in serum, urine and tissues of mice given a 50 mg/kg sc dose.

Body fluid or tissue	Concentration (μg/ml[a] or μg/g) at minute					
	15	30	60	120	240	360
Serum	58.8	55.7	21.1	3.25	0.22	0.12
Urine			46.3[b]	50.8[b]	64.3[b]	61.0[b]
Kidney			34.7	5.6	0.7	0.4
Liver			19.5	2.7	0.4	0.8
Lung			7.3	0.6	<0.5	<0.5
Muscle			2.9	<0.5	<0.5	<0.5
Bone (femur)			0.8	0.3	<0.3	<0.3
Brain			<0.5	<0.5	<0.5	<0.5

Serum parameters: AUC=51.7 μg·hour/ml, $T_{1/2}$=31.3 minutes.

[a] Determined by bioassay; sensitivity of the assay was 0.1 μg/ml.

[b] Percent dose recovered.

Table 5. Effect of probenecid[a] on the pharmacokinetics of L-658,310 in mice given a 20 mg/kg sc dose.

Time (hours)	Serum		Time (hours)	Urine	
	Concentration (μg/ml)[b]			Dose recovered (%)	
	L-658,310	L-658,310+ BEN		L-658,310	L-658,310+ BEN
0.25	23.8	27.0			
0.5	18.8	19.3			
1	9.3	9.0	0~1	46.5	23.4
2	1.1	1.2	0~2	45.5	63.9
4	<0.1	<0.1	0~4	53.6	78.8
6	<0.1	<0.1	0~6	62.0	73.3
AUC (μg·hour/ml)	19.4	20.3			
$T_{1/2}$ (minutes)	19.2	20.3			

[a] Probenecid (BEN), 500 mg/kg, was administered by gavage immediately before the L-658,310 dose.

[b] Determined by bioassay; sensitivity of the assay was 0.1 μg/ml.

ceftazidime after a 20 mg/kg sc dose to mice were: Cmax=21.1 μg/ml; Tmax=15 minutes; AUC=16.5 μg·hour/ml; and $T_{1/2}$=23 minutes. Urinary recovery was about 60% in 6 hours.

Following the 50 mg/kg dose, L-658,310 was detected in all tissues examined except brain (Table 4). At 1 hour after dosing, the concentration in kidney was greater than that in serum, and that in liver approximated the serum concentration. Lesser amounts were found in lung, muscle and bone. Thereafter, concentrations in tissues declined at about the same rate as the serum concentrations.

Urinary excretion of L-658,310 appeared to be mainly by glomerular filtration since concomitant administration of a 500 mg/kg oral dose of probenecid did not significantly increase serum concentrations nor did it extend the $T_{1/2}$ of L-658,310 in serum of mice (Table 5). The AUC and $T_{1/2}$ were essentially the same for both groups. Probenecid did delay urine output for about 1 hour, but urinary recovery of dose in 6 hours was actually greater in mice given probenecid than in the controls.

Pharmacokinetics in Monkeys

L-658,310 was also absorbed efficiently from the im injection site in rhesus monkeys and reached peak serum concentrations of 27.5~28.8 μg/ml at 30 to 60 minutes after a 10 mg/kg dose and fell to 0.9~1.6 μg/ml at 6 hours (Fig. 2). The AUC averaged 70 μg·hour/ml and the $T_{1/2}$ was 77 minutes. As in mice, L-658,310 was excreted rapidly into urine of monkeys and concentrations of 400 μg/ml

Fig. 2. Concentrations of L-658,310 in serum of rhesus monkeys given a 10 mg/kg dose by the im route.

● Monkey 83-233, ■ monkey 83-234, ▲ monkey 83-235.

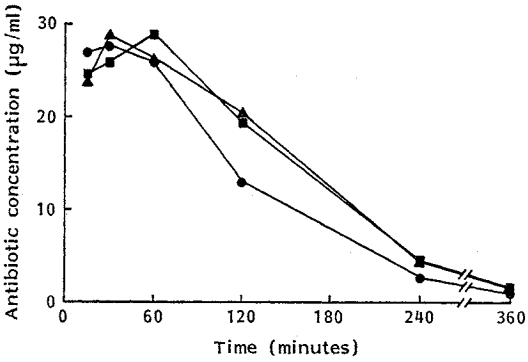
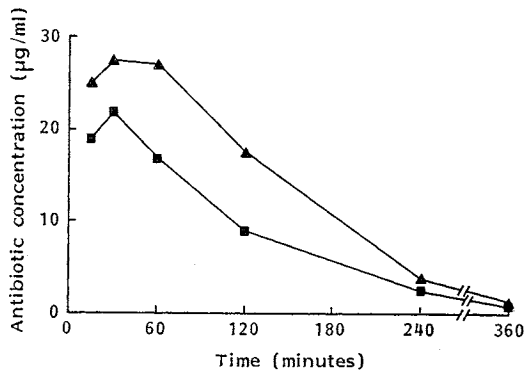


Fig. 3. Mean concentrations of L-658,310 and ceftazidime in serum of rhesus monkeys given 10 mg/kg by the im route in a crossover design.



Dose	Cmax (μg/ml)	AUC (μg·hour/ml)	$T_{1/2}$ (minutes)
L-658,310 (▲)	27.4	70	77
Ceftazidime (■)	21.8	45	74

or higher were found in the 0~6 hours samples. Urinary recovery of administered dose was greater than 99% in 24 hours.

The pharmacokinetics of L-658,310 in monkeys were, as in mice, similar to those of ceftazidime (Fig. 3). The mean peak serum concentration of L-658,310 was higher than that of ceftazidime, 27.4 μg/ml compared to 21.8 μg/ml for ceftazidime at 30 minutes after dosing, and serum levels of L-658,310 remained higher than those of ceftazidime for at least 2 hours after dosing. Consequently, the AUC for L-658,310 was greater than that of ceftazidime: 70 vs. 45 μg·hour/ml. The $T_{1/2}$, however, was similar: 77 minutes for L-658,310 and 74 minutes for ceftazidime.

Like L-658,310, ceftazidime was excreted rapidly into urine of monkeys. In two of the three monkeys, 100% of the dose was recovered in 24 hours. Only 63% of the dose was recovered in urine of the third monkey. We have no explanation for this finding.

Binding to Human Plasma Proteins

Binding of L-658,310 to human plasma proteins was relatively constant, 46~50%, at concentrations ranging from 25~100 μg/ml. Binding of ceftazidime was only 16% when tested at a concentration of 100 μg/ml, which is in agreement with data reported by HARDING *et al.*[6].

Discussion

Chemotherapeutic studies in mice have demonstrated a potential for L-658,310 as an effective agent against bacteremias due to Gram-negative pathogens in humans, even in those with underlying diseases. The especially potent activity of L-658,310 against strains of *P. aeruginosa* that are resistant to many of the presently available antibacterial agents and its stability to β-lactamases make it an attractive candidate for human trials.

The pharmacokinetic profile of L-658,310 in both mice and monkeys is similar to that of ceftazidime. In monkeys given a 10 mg/kg im dose, serum levels were above the MICs of most susceptible Gram-negative pathogens for at least 3 hours and high concentrations of drug were present in urine for 6 hours after dosing. Elimination of L-658,310 appears to be mainly by glomerular filtration since concomitant administration of probenecid had little or no effect on the pharmacokinetics of

L-658,310 in mice. Studies in human volunteers have shown that ceftazidime is eliminated primarily by glomerular filtration and that the influence of probenecid was insignificant[7]. Our studies in laboratory animals have demonstrated many similarities in the pharmacokinetics of L-658,310 and ceftazidime and suggest a moderately long half-life for L-658,310 in humans, at least as long as that reported for ceftazidime, *i.e.* about 1.7 hours[6,7].

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