L-658,310, A NEW INJECTABLE CEPHALOSPORIN

II. *IN VITRO* AND *IN VIVO* INTERACTIONS BETWEEN L-658,310 AND VARIOUS AMINOGLYCOSIDES OR CIPROFLOXACIN *VERSUS* CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA*†

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Combinations of L-658,310 and an aminoglycoside or ciprofloxacin were tested against clinical isolates of *Pseudomonas aeruginosa* using a checkerboard broth dilution technique. Using the mean fractional bactericidal concentration of ≤ 0.5 as the criterion for synergy, the combinations L-658,310/tobramycin and L-658,310/ciprofloxacin against strains of *P. aeruginosa* resistant to the companion drug were synergistic. The data plotted as isobolograms showed synergy for all combinations tested. Synergy was clearly demonstrated in time-kill experiments. A greater than 3-log decrease in viable cell count for *P. aeruginosa* was seen after exposure for 24 hours to subinhibitory concentrations of the combined agents. In *in vivo* mouse models, the efficacy of L-658,310 against experimental *P. aeruginosa* bacteremias was enhanced by the addition of a low dose of an aminoglycoside to the treatment regimen, thus confirming the synergy demonstrated in time-kill experiments.

L-658,310, a new semisynthetic cephalosporin, synthesized at Okazaki Research Laboratories, Banyu Pharmaceutical Co., Ltd., Okazaki, Japan, is a broad spectrum antibacterial agent that is especially potent against *Pseudomonas* species[2~4]. Although L-658,310 is sufficiently potent to permit monotherapy in experimental infections in laboratory animals, it was of interest to investigate the interaction of L-658,310 with some other agents used in the treatment of *Pseudomonas* infections. To study the interaction we selected checkerboard tests, time-kill experiments, and mouse protection tests using clinical isolates of *Pseudomonas aeruginosa* that were both resistant and susceptible to the second agents.

Materials and Methods

Antibiotics

Laboratory powders of L-658,310 were used for these studies. All were >99% pure and were readily soluble in 0.07 M SORENSEN's buffer pH 7. Ciprofloxacin was kindly provided by Miles Pharmaceuticals, West Haven, Ct., and gentamicin by Schering Corporation, Bloomfield, NJ. Two batches of amikacin were used: One was supplied by Bristol Laboratories, Syracuse, NY, and the second was purchased from Sigma Chemical Company, St. Louis, MO. There were also two sources of tobramycin: The first was a solution provided by Eli Lilly and Company, Indianapolis, IN, and the second was purchased as a powder from Sigma. Stock solutions of the aminoglycosides and ciprofloxacin were prepared in sterile distilled water for each test and diluted in water or broth as required. All antibacterials were adjusted for potency.

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Cultures

The strains of *P. aeruginosa* are recent clinical isolates selected for resistance or susceptibility to ciprofloxacin or the aminoglycosides. The identifying numbers are those of the Merck Clinical Microbiology Services Laboratory. They were maintained in the lyophilized state or on Trypticase Soy agar (TSA) (BBL) slants stored at 5°C.

Animals

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

Checkerboard Technique

Checkerboard tests were performed using a microtiter broth dilution procedure. Each agent was tested alone and in combination with L-658,310 using a checkerboard microtiter broth dilution procedure. The test medium employed was Mueller-Hinton (MH) broth (Difco) supplemented with $CaCl_2$ (50 μg/ml) and $MgCl_2$ (25 μg/ml) as recommended by the NCCLS for broth dilution procedures especially when using aminoglycosides and *P. aeruginosa*[5].

Each agent was tested alone and in combination with L-658,310 against strains of *P. aeruginosa* selected for susceptibility and resistance to the aminoglycosides and ciprofloxacin.

Amikacin, gentamicin, and ciprofloxacin initially were dissolved in water. Tobramycin was available as an aqueous solution, and L-658,310 was dissolved in SORENSEN's 0.07 M phosphate buffer (pH 7). After filter sterilization, the agents were diluted with MH broth to achieve the top levels to be used alone or in combination. Subsequent serial 2-fold dilutions were prepared with broth using a Cetus Pro/Pette.

Overnight cultures of the test strains were diluted with MH broth, and 1.5 μl of the test culture were added to 100 μl of the diluted compound(s) in the test wells using a Dynatech MIC 2000 Inoculator. This resulted in a final inoculum of about 1×10^5 cfu/ml.

After incubation at 35°C for 20 hours, the test wells were examined for growth and the MIC was recorded as the lowest level of compound that allowed no visible growth. At this time, 1.5 μl from each well were transferred to the surface of drug-free MH agar using a Dynatech MIC 2000 Inoculator. After incubation at 35°C for 20 hours, the MBC was recorded as the lowest concentration of antibiotic showing 5 or less discrete colonies indicating that at least 97% of the initial inoculum had been killed.

In order to determine whether the drug combinations were synergistic, additive, or antagonistic, a mean fractional inhibitory concentration ($\overline{FIC}$) index was calculated for each set of data according to FOCK *et al.*[6]. The FIC for each drug was calculated by dividing the concentration of drug in the combination by the MIC of the drug alone. The index is the summation of these values (Equation 1). The mean FIC index equals the sum of the indices divided by the number of combinations evaluated (Equation 2).

$$FIC=\frac{[A]}{MIC_A}+\frac{[B]}{MIC_B} \qquad (1)$$

$$\overline{FIC}=\sum_1^n FIC/n \qquad (2)$$

The mean fractional bactericidal concentration ($\overline{FBC}$) was calculated in the same manner. A mean FIC or FBC index of ≤0.5 denoted synergy, >0.5 but <2.0 denoted an additive effect, and ≥2.0 indicated antagonism.

Time-kill Experiments

Time-kill experiments were performed in which the number of cfu was determined after exposure to the agents alone or in combination. Tubes containing one part of drug or mixtures of drugs were inoculated with nine parts of supplemented MH broth seeded with the test culture resulting in approximately 1×10^5 cfu/ml. The inoculated tubes were incubated at 35°C and aliquots were removed at 3, 5, 7 and 24 hours for plate counting. After incubation at 35°C for at least 48 hours, colonies

were counted and the number of cfu/ml broth was calculated. In these tests synergy was defined as a >3 $\log_{10}$ decrease in cfu/ml between the combination and its more active constituent after 24 hours.

Chemotherapeutic Studies

Experimental systemic bacteremias were produced in CD-1 female mice (average weight 21 g) by ip injection of 0.5 ml of a suitable dilution of an overnight broth culture of the pathogen in 5% hog gastric mucin (Wilson Laboratories, Division of Inolex Corp., Park Forest South, IL, U.S.A.). The challenge dose ranged from 32~91 times the LD_{50}.

The aminoglycosides and L-658,310 were solubilized as for *in vitro* studies and were administered singly or in combination in a volume of 0.25 ml by the sc route immediately after infection and again 6 hours later. There were ten mice at each treatment level. In combination, the dose for gentamicin, tobramycin and amikacin was held constant at 12.5 or 25 mg/kg, and L-658,310 was titrated in 2-fold increments over a broad range. Separate sc injection sites were used for each antibiotic. The mice were observed for 7 days and the number of survivors was used to calculate the median effective dose (ED_{50}) by the method of KNUDSEN and CURTIS[7].

Results and Discussion

Checkerboard Tests

With strains of *P. aeruginosa* selected for resistance to the second agent, a synergistic effect based on the mean FBC was observed only for the combinations of L-658,310 with tobramycin and ciprofloxacin (Table 1). The mean FBC was 0.5 in each case. Using the most rigorous definition for synergy, the checkerboard test results were marginal; however, if synergy is defined as the sum of the fractions equal to or less than one[8], then all combinations were synergistic. These results are best illustrated as isobolograms (Fig. 1). In all four examples the curve bows inward toward the x and y axes indicating synergy.

With strains of *P. aeruginosa* susceptible to the second agent, no distinct synergy was seen in the checkerboard tests. Mean FBC values ranged from 0.79 to 1.06 (Table 2). However when isobolograms were plotted for the combinations of amikacin or gentamicin with L-658,310 (Fig. 2) synergy was indicated. In no case was antagonism observed.

Time-kill Experiments

Fig. 3 shows the results with L-658,310 plus gentamicin or tobramycin against *P. aeruginosa*

Table 1. Effect of combinations of L-658,310 and aminoglycosides or ciprofloxacin against resistant clinical isolates of *Pseudomonas aeruginosa*.

P. aeruginosa CL No.	Antimicrobial agents	Alone (μg/ml)		Combined index	
		MIC[a]	MBC[b]	$\overline{FIC}$	$\overline{FBC}$
2411	L-658,310	1.0	4.0	0.88	0.85
	Amikacin	32.0	32.0		
3092	L-658,310	0.5	1.0	0.56	0.56
	Gentamicin	16.0	16.0		
2049	L-658,310	0.125	0.5	0.70	0.50
	Tobramycin	64.0	128.0		
2824	L-658,310	2.0	4.0	0.70	0.50
	Ciprofloxacin	8.0	16.0		

[a] Mueller-Hinton broth, inoculum: 10^5 cfu/ml, incubation: 18~22 hours at 35°C, endpoint: no visible growth.

[b] Drug-free Mueller-Hinton agar, incubation: ~24 hours, endpoint: $\leq$5 discrete colonies ($\geq$97% kill).

Fig. 1. *In vitro* combinations of L-658,310 (L) and amikacin (A), gentamicin (G), tobramycin (T) or ciprofloxacin (C) against clinical isolates of *Pseudomonas aeruginosa* resistant to the second agent.

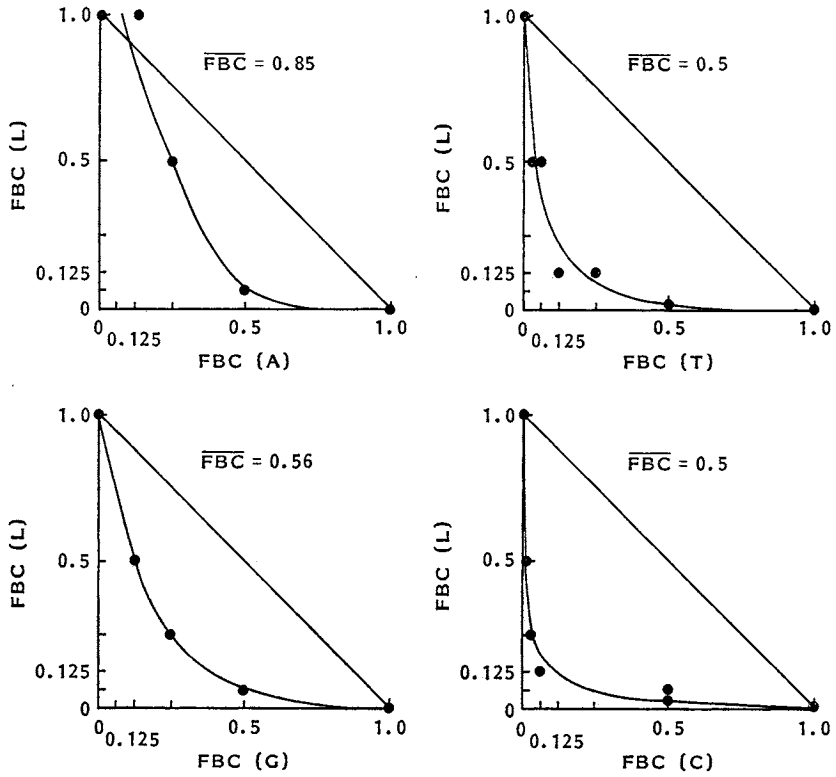


Table 2. Effect of combinations of L-658,310 and aminoglycosides or ciprofloxacin against susceptible clinical isolates of *Pseudomonas aeruginosa*.

P. aeruginosa CL No.	Antimicrobial agents	Alone (μg/ml)		Combined index	
		MIC[a]	MBC[b]	$\overline{FIC}$	$\overline{FBC}$
665	L-658,310	1.0	2.0	0.97	0.81
	Amikacin	4.0	8.0		
665	L-658,310	1.0	2.0	0.88	0.79
	Gentamicin	2.0	4.0		
665	L-658,310	1.0	2.0	0.93	0.92
	Tobramycin	1.0	1.0		
2823	L-658,310	1.0	2.0	1.43	1.06
	Ciprofloxacin	0.5	1.0		

[a,b] See footnotes to Table 1.

strains resistant to the aminoglycosides. In both cases the number of viable cells 5 hours after exposure to the combination was at least 10-fold lower than the more effective agent alone: In 24 hours a >3 $\log_{10}$ decrease was seen, clearly denoting synergy. Similar results were seen with L-658,310 and amikacin where 1/4 of their individual bactericidal levels were bactericidal in combination. When 1/32 the ciprofloxacin MBC was combined with 1/8 the MBC of L-658,310, synergy was not seen until about the 7th-hour (Fig. 4).

Results obtained against the antibiotic susceptible strains varied. The two examples shown in

Fig. 2. *In vitro* combinations of L-658,310 (L) and amikacin (A) or gentamicin (G) against clinical isolates of *Pseudomonas aeruginosa* susceptible to the second agent.

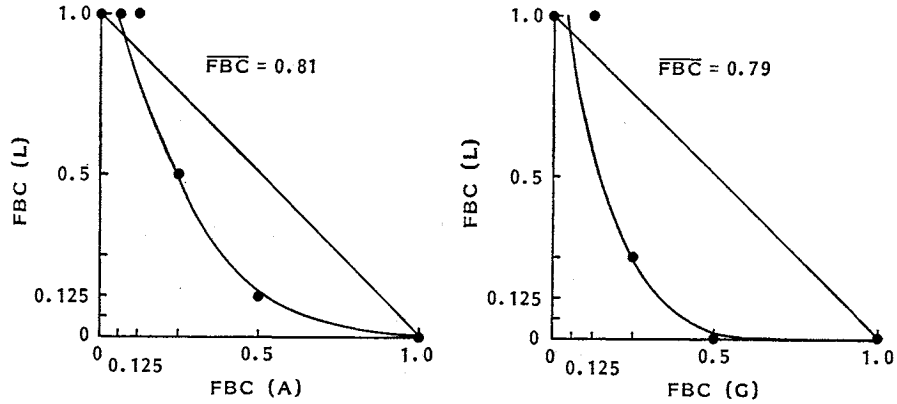
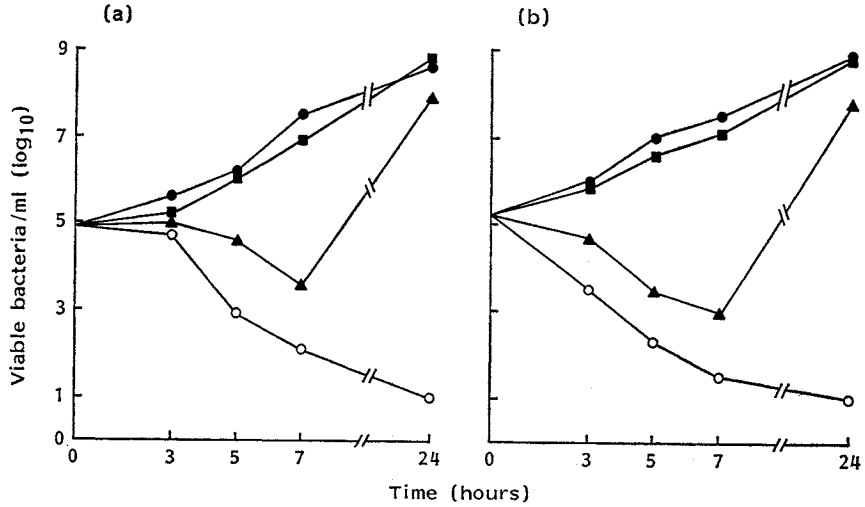


Fig. 3. *In vitro* combinations of L-658,310 (L) and gentamicin (G) or tobramycin (T) against clinical isolates of *Pseudomonas aeruginosa*.



(a) *P. aeruginosa* CL 3092 gentamicin-resistant.
● Control, ▲ (L) 1/8 MBC, ■ (G) 1/8 MBC, ○ (G) 1/8 MBC+(L) 1/8 MBC.
(b) *P. aeruginosa* CL 2049 tobramycin-resistant.
● Control, ▲ (L) 1/4 MBC, ■ (T) 1/8 MBC, ○ (T) 1/8 MBC+(L) 1/4 MBC.

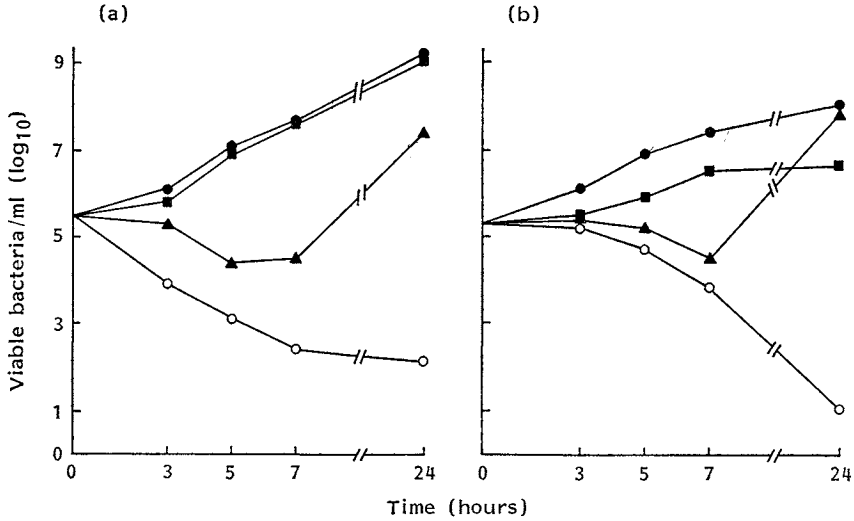
Fig. 5 illustrate that the combinations of gentamicin and amikacin with L-658,310 were synergistic, although neither combination resulted in a total cidal effect. Similar results were seen for combinations of tobramycin and ciprofloxacin with L-658,310 (data not shown).

In all cases the antibiotic concentrations used were within the range likely to be present in serum of patients under therapy.

In Vivo Studies

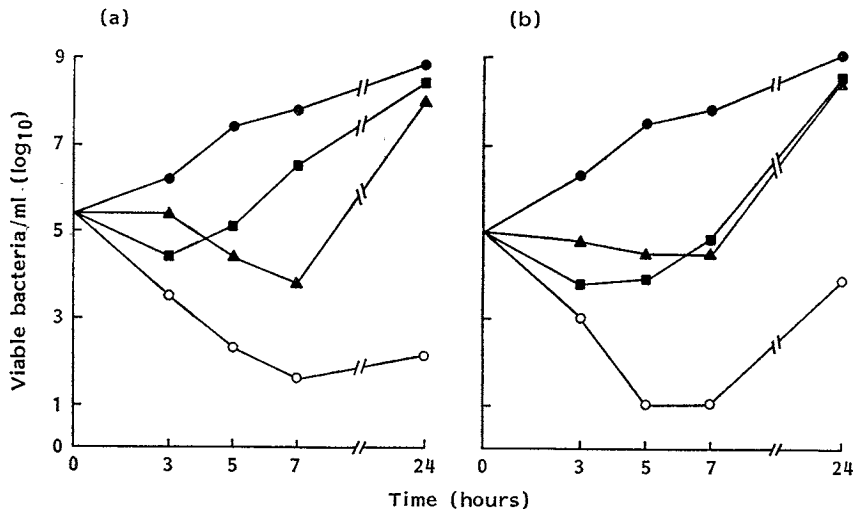
In Table 3, the data show that L-658,310 and gentamicin protected mice against *P. aeruginosa* CL 3092 when each agent was used alone. The ED_{50}s were 30.4 mg/kg×2 sc doses for L-658,310 and

Fig. 4. *In vitro* combinations of L-658,310 (L) and amikacin (A) or ciprofloxacin (C) against clinical isolates of *Pseudomonas aeruginosa*.



(a) *P. aeruginosa* CL 2411 amikacin-resistant.
● Control, ▲ (L) 1/4 MBC, ■ (A) 1/4 MBC, ○ (A) 1/4 MBC+(L) 1/4 MBC.
(b) *P. aeruginosa* CL 2824 ciprofloxacin-resistant.
● Control, ▲ (L) 1/8 MBC, ■ (C) 1/32 MBC, ○ (C) 1/32 MBC+(L) 1/8 MBC.

Fig. 5. *In vitro* combinations of L-658,310 (L) and gentamicin (G) or amikacin (A) against a susceptible clinical isolate of *Pseudomonas aeruginosa*.



(a) *P. aeruginosa* CL 665 gentamicin-susceptible.
● Control, ▲ (L) 1/4 MBC, ■ (G) 1/8 MBC, ○ (G) 1/8 MBC+(L) 1/4 MBC.
(b) *P. aeruginosa* CL 665 amikacin-susceptible.
● Control, ▲ (L) 1/4 MBC, ■ (A) 1/4 MBC, ○ (A) 1/4 MBC+(L) 1/4 MBC.

63.3 mg/kg×2 sc doses for gentamicin. When L-658,310 was administered along with 12.5 mg/kg gentamicin, the ED_{50} of L-658,310 was 2.0 mg/kg. When the gentamicin dose was increased to 25 mg/kg, the ED_{50} of L-658,310 was <1.6 mg/kg×2 sc doses. The actual ED_{50} could not be calculated

Table 3. Efficacy of L-658,310 alone and in combination with gentamicin (GEN) against *Pseudomonas aeruginosa* CL 3092 bacteremia[a] in mice.

Antibacterial therapy[b]	MIC (μg/ml)[c]	ED_{50} (mg/kg×2 sc doses)[d]
L-658,310	0.5	30.4
GEN	16	63.3
L-658,310+ GEN (12.5 mg/kg)		2.0
L-658,310+ GEN (25 mg/kg)		<1.6

[a] Infection was established by ip injection of the pathogen in 5% hog gastric mucin. The challenge contained *ca.* 32 LD_{50} doses.
[b] Therapy was by the sc route at 0 and 6 hours after infection.
[c] Determined in an agar dilution assay.
[d] ED_{50}, median effective dose, calculated on the seventh day post infection.

Table 4. Efficacy of L-658,310 alone and in combination with tobramycin (TOB) against *Pseudomonas aeruginosa* CL 2049 bacteremia[a] in mice.

Antibacterial therapy[b]	MIC (μg/ml)[c]	ED_{50} (mg/kg×2 sc doses)[d]
L-658,310	0.125	12.23
TOB	64	149.61
L-658,310+ TOB (12.5 mg/kg)		4.61
L-658,310+ TOB (25 mg/kg)		2.43

[a] Infection was established by ip injection of the pathogen in 5% hog gastric mucin. The challenge contained *ca.* 91 LD_{50} doses.
[b~d] See footnotes to Table 3 above.

Table 5. Efficacy of L-658,310 alone and in combination with amikacin (AMI) against *Pseudomonas aeruginosa* CL 2411 bacteremia[a] in mice.

Antibacterial therapy[b]	MIC (μg/ml)[c]	ED_{50} (mg/kg×2 sc doses)[d]
L-658,310	1	90.9
AMI	32	87.2
L-658,310+ AMI (12.5 mg/kg)		34.0
L-658,310+ AMI (25 mg/kg)		19.9

[a] Infection was established by ip injection of the pathogen in 5% hog gastric mucin. The challenge contained *ca.* 32 LD_{50} doses.
[b~d] See footnotes to Table 3 above.

because >95% of the infected, treated mice were alive on the seventh day after infection. Thus, efficacy of L-658,310 was enhanced 15-fold by addition of a low dose of gentamicin to the treatment regimen and >19-fold when the higher dose was given.

Two other combinations, L-658,310/amikacin and L-658,310/tobramycin were also more efficacious than either agent alone when given to mice experimentally infected with *P. aeruginosa* CL 2411 or *P. aeruginosa* CL 2049. It can be seen in Tables 4 and 5 that the ED_{50} of L-658,310 was reduced by a factor of about 5 when the aminoglycoside was administered at 25 mg/kg, and by a factor of 2.6 when the aminoglycoside dose was 12.5 mg/kg. In both cases the 25 mg/kg dose was well below the ED_{50} value of either aminoglycoside administered alone.

P. aeruginosa CL 2824, used for the L-658,310 plus ciprofloxacin *in vitro* interaction studies, was not mouse virulent, thus this combination could not be evaluated *in vivo*.

In summary, L-658,310, a novel cephalosporin, is capable of potentiating the activity of gentamicin, tobramycin, amikacin and ciprofloxacin *in vitro* against strains of *P. aeruginosa* resistant to the companion drugs. It did not antagonize the activities of the companion drugs against susceptible strains of *P. aeruginosa*. In most cases, synergy *in vitro* between L-658,310 and the companion drugs was best illustrated in time-kill curves using concentrations of the antibiotics that are achievable in the serum of patients. Enhanced rates of killing *in vitro* were good predictors of improved efficacy in animal protection tests.

References

1) VALIANT, M.; E. GILFILLAN & B. PELAK: *In vitro* and *in vivo* synergy between L-658,310, a cephalosporin,

and various aminoglycosides or ciprofloxacin vs clinical isolates of *Pseudomonas aeruginosa*. Program and Abstracts of the 27th Intersci. Conf. on Antimicrob. Agents Chemother., No. 808, p. 237, New York, Oct 4~7, 1987

2) NAKAGAWA, S.; M. SANADA, K. MATSUDA, N. HAZUMI & N. TANAKA: Biological activity of BO-1236, a new antipseudomonal cephalosporin. Antimicrob. Agents Chemother. 31: 1100~1105, 1987
3) WEISSBERGER, B. A.; G. K. ABRUZZO, R. A. FROMTLING, C. GILL, S. PONTICAS, M. E. VALIANT, D. L. SHUNGU & H. H. GADEBUSCH: L-658,310, a new injectable cephalosporin. I. *In vitro* antibacterial properties. J. Antibiotics 42: 795~806, 1989
4) GILFILLAN, E. C.; B. A. PELAK, B. A. WEISSBERGER, G. K. ABRUZZO, R. A. FROMTLING, J. A. BLAND, S. K. HADLEY & H. H. GADEBUSCH: L-658,310, a new injectable cephalosporin. III. Experimental chemotherapeutics and pharmacokinetics in laboratory animals. J. Antibiotics 42: 815~822, 1989
5) National Committee for Clinical Laboratory Standards: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. NCCLS Document M7-A, Vol. 5, No. 22, National Committee for Clinical Laboratory Standards, Villanova, 1985
6) FOCK, R. R. E.; B. THORMÄHLEN, C. STELZL & R. LAUFS: Combination of β-lactam antibiotics in vitro. *In* Proc. 13th. Int. Congr. Chemother., *Eds.*, K. H. SPITZY & K. KARRER, Part 48., No. 1, pp. 1~4, Vienna, 1983
7) KNUDSEN, L. F. & J. M. CURTIS: The use of the angular transformation in biological assays. J. Am. Stat. Assoc. 42: 282~296, 1947
8) SCHOENKNECHT, F. D.; L. D. SABATH & C. THORNSBERRY: Susceptibility tests: Special tests. *In* Manual of Clinical Microbiology. 4th Ed. *Ed.*, E. H. LENNETTE *et al.*, pp. 1000~1008, American Society for Microbiology, Washington, D.C., 1985