ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1976, p. 589-594

Vol. 9, No. 4
Printed in U.S.A.

In Vivo Synergy Between 6β-Amidinopenicillanic Acid Derivatives and Other Antibiotics

E. GRUNBERG,* R. CLEELAND, G. BESKID, AND W. F. DELORENZO

Research Division, Hoffmann-La Roche Inc., Nutley, New Jersey 07110

Received for publication 16 December 1975

Both an oral and a parenteral form of a 6β-amidinopenicillanic acid derivative were found to have appreciable activity against gram-negative bacteria and poor activity against gram-positive bacteria in vivo. When administered orally or parenterally, definite synergy was demonstrated between the amidinopenicillins and ampicillin, amoxicillin, benzylpenicillin, cefazolin, or carbenicillin in infections with a number of gram-negative bacteria, including *Klebsiella, Enterobacter, Escherichia, Proteus, Salmonella,* and *Haemophilus* species in mice. Synergy was also observed between the parenteral amidinopenicillin and benzylpenicillin in the *Staphylococcus aureus* infection but not in infections with other gram-positive organisms. No synergy was demonstrated between the parenteral amidinopenicillin and erythromycin or oxytetracycline in infections with gram-positive or gram-negative organisms. Synergy between the parenteral amidinopenicillin and gentamicin was observed only in the case of *Escherichia coli.*

An oral form and a parenteral form of a 6β-amidinopenicillanic acid derivative were obtained from Leo Pharmaceuticals (Copenhagen, Denmark) for chemotherapeutic evaluation. As has been reported in the literature (5) and confirmed in our laboratory, these two derivatives had antibacterial spectra, both in vitro and in vivo, quite different from that of benzylpenicillin (i.e., appreciable activity against gram-negative bacteria, especially *Escherichia coli,* but poor activity against gram-positive bacteria).

The amidinopenicillins had been shown to have a mode of action different from that of other β-lactam antibiotics (2-4, 6-8). In three recent reports (11; H. C. Neu, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 15th, Washington, D.C., Abstr. 145, 1975; R. Baltimore, J. O. Klein, C. Wilcox, and M. Finland, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 15th, Washington, D.C., Abstr. 140, 1975), it has been shown that the parenteral amidinopenicillin FL 1060, when combined with aminopenicillins or cephalosporins, exerted in vitro a definite synergistic effect against a number of strains of *Enterobacteriaceae.*

The present study was undertaken to determine the synergistic potential of these two amidinopenicillins when combined with benzylpenicillin, amoxicillin, ampicillin, carbenicillin, and cefazolin as well as with erythromycin, gentamicin, and oxytetracycline against gram-negative and gram-positive bacterial infections in mice.

The results of these studies are reported herein.

MATERIALS AND METHODS

Antibiotics. Benzylpenicillin, amoxicillin, ampicillin, carbenicillin, cefazolin, erythromycin, gentamicin, and oxytetracycline were all suspended or dissolved in distilled water to yield concentrations calculated on the basis of the free base. The two 6β-amidinopenicillanic acid derivatives (FL1060 and FL1039, Leo Pharmaceuticals) were treated in the same manner as the above agents.

Bacteria. The bacteria employed in this study included the following: *Escherichia coli* (three strains); *Proteus mirabilis* (two strains); *Proteus vulgaris* (two strains); *Klebsiella pneumoniae* (five strains); *Enterobacter cloacae; Salmonella typhosa; Salmonella schottmuelleri; Serratia marcescens* (two strains); *Haemophilus influenzae* (four strains); *Pseudomonas aeruginosa* (two strains); *Staphylococcus aureus; Streptococcus pyrogenes;* and *Streptococcus pneumoniae.*

All bacterial cultures were grown in Trypticase soy broth (BBL) with the exception of the *H. influenzae* strains, which were propagated in brain heart infusion broth (BBL) containing 2.5% supplement C (Difco). For the cultivation of *S. pneumoniae* and *S. pyogenes,* Trypticase soy broth was supplemented with 10% goat serum.

Infection. Experimental infections were carried out in Swiss albino mice weighing 18 to 20 g. The animals were infected intraperitoneally with 0.5 ml

of appropriate diluted suspensions of overnight cultures containing 100 to 1,000 minimal lethal doses. Infecting inocula for *S. pneumonia, S. pyogenes,* and *K. pneumoniae* were diluted in Trypticase soy broth, whereas inocula for the four *H. influenzae* strains were prepared in 2.5% mucin. The infecting inoculum for each of the other bacterial strains was prepared in 5% mucin.

Treatment. The animals were treated subcutaneously or orally with graded doses of the antibiotics in 1.0-ml amounts. Ten animals were used for each dosage level. Where subcutaneous and oral treatments were administered in combination, the amount for each route was contained in 0.5 ml instead of 1.0 ml. The treatment schedule for administration of the single agents or combinations was either (i) one treatment given immediately after infection or (ii) two treatments, one administered immediately after infection and the second 5 h later.

The number of mice surviving for 14 days was used to calculate the 50% protective dose (PD_{50}) by the method of Reed and Muench (9).

RESULTS

Shown in Fig. 1 and 2 are the structures for FL1060 and FL1039, which are derivatives of 6β-amidinopenicillanic acid, in contrast to other penicillins, which are derivatives of 6-aminopenicillanic acid. FL1060, the parenteral form, is poorly absorbed when administered orally. The pivaloyloxymethyl ester of FL1060, designated FL1039, is well absorbed orally and is rapidly hydrolyzed enzymatically, resulting in complete conversion to FL1060 in the blood and urine (10). Both compounds are readily soluble in water and stable at concentrations of 10 mg/ml for at least 1 month at 4 C.

In Table 1 are shown results of tests to determine the in vivo activity of ampicillin, amoxicillin, and FL1060 alone and the synergistic effect of a 1:1 fixed combination of these agents against 20 gram-negative bacterial strains. Synergy in vivo was considered to have occurred if the fractional inhibitory concentration index was equal to or less than 0.5. The fractional inhibitory concentration index was calculated by dividing the PD_{50} value obtained for each of the components in the combination by the PD_{50} value for each component alone and adding the two quotients.

Combination of FL1060 with both amoxicillin and ampicillin resulted in definite synergistic effects in the case of the two *E. coli* strains, three of the four *Proteus* strains, three of the five *Klebsiella* strains, *E. cloacae, Salmonella typhosa, S. schottmuelleri,* and the three *H. influenzae* strains. In the case of *K. pneumoniae* 7192, synergy was seen with the combination of amoxicillin and FL1060 but not with ampicillin and FL1060. In the case of *S. marcescens* S966, synergy was seen with the combination of ampicillin and FL1060 but not with amoxicillin and FL1060. In only four of the 20 infections was no synergy demonstrated (i.e., *P. vulgaris* ATCC 6380, *K. pneumoniae* PM, *S. marcescens* S714, and *P. aeruginosa* B). In those infections where amoxicillin and ampicillin were administered in combination, a synergistic effect was only observed in the case of *H. influenzae* 9B.

Shown in Table 2 are the results of studies in which the route of treatment was varied and both the parenteral (FL1060) and oral (FL1039) 6β-amidinopenicillins were employed. Thus, ampicillin was combined with FL1039 and administered orally, or ampicillin was given subcutaneously and FL1039 was given orally. Alternatively, ampicillin was combined with FL1060 and administered subcutaneously, or ampicillin was given orally and FL1060 was given subcutaneously. The same schedule was followed when amoxicillin was combined with FL1060 and FL1039. In the combination of ampicillin with amoxicillin, the two antibiotics were given simultaneously by either the subcutaneous route or the oral route only. The amidinopenicillins acted synergistically with ampicillin and amoxicillin in the *E. coli, P. vulgaris, K. pneumoniae* A and 1, and *E. cloacae* infections by all routes tested. In the *P. mirabilis* infection, FL1039 acted synergistically with both ampicillin and amoxicillin when treatment was administered orally. FL1060 acted synergistically with both ampicillin and amoxi-

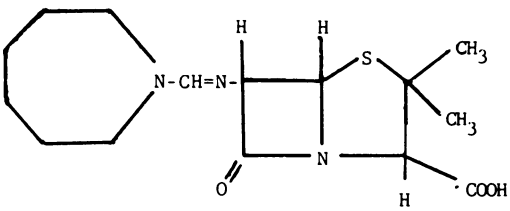

FIG. 1. *Structure of parenteral amidinopenicillin (FL1060).*

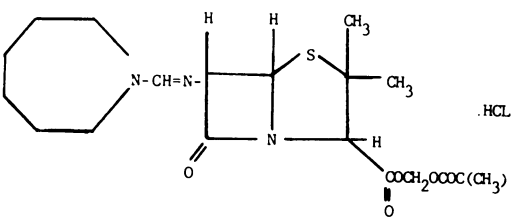

FIG. 2. *Structure of oral amidinopenicillin (FL1039).*

TABLE 1. *Effect of administering ampicillin, amoxicillin, and FL1060 alone and combined in a 1:1 ratio subcutaneously[a] against gram-negative bacterial infections in mice*

Organism	PD_{50} (mg/kg)						FIC index[b]		
	Ampicillin	Amoxicillin	FL1060	Ampicillin + FL1060[c]	Amoxicillin + FL1060[c]	Ampicillin + amoxicillin[c]	Ampicillin + FL1060	Amoxicillin + FL1060	Ampicillin + amoxicillin
E. coli 503-455	6.2	1.1	8.8	<0.5	<0.5	1.3	≤0.14	≤0.51	>1
E. coli 5152	>500	>500	>250	82	61	>500	≤0.49	≤0.37	>1
P. mirabilis 2	2.9	1.5	75	<0.5	<0.5	0.84	≤0.18	≤0.34	0.85
P. mirabilis 8026	371	>500	>250	57	55	339	≤0.38	≤0.33	>1
P. vulgaris 100	71	72	295	3.3	14	141	0.06	0.24	>1
P. vulgaris ATCC 6380	343	>500	6.5	23	5.3	>500	>1	≤0.83	>1
K. pneumoniae A	32	7.4	>50	5	3.0		≤0.26	≤0.47	
K. pneumoniae 1	254	142	11	1.6	1.4		0.15	0.14	
K. pneumoniae 503-516	20	27	33	2.2	2		0.18	0.14	
K. pneumoniae 7192	50	136	>250	26	4.3	25	≤0.62	≤0.05	0.68
K. pneumoniae PM	118	148	>500	88	109	110	≤0.93	≤0.96	>1
E. cloacae 503-964	6.8	10	34	1.6	2.2		0.28	0.29	
S. typhosa P58A	1.6	1.4	0.9	<0.1	0.18		≤0.17	0.33	
S. schottmuelleri	2.5	3.9	5.6	0.43	0.42		0.25	0.18	
S. marcescens S966	81	47	>500	37	31		≤0.53	≤0.72	
S. marcescens S714	28	16	42	21	13		>1	>1	
H. influenzae 6	8.7	8	65	1.3	0.8	2.5	0.17	0.11	0.60
H. influenzae 9B	61	24	134	5.2	6.3	9	0.12	0.31	0.52
H. influenzae H54	36	36	>250	11	5.6	16	≤0.35	≤0.18	0.89
P. aeruginosa B	>500	>500	>500	~500	>500	500	>1	>1	>1

[a] Two treatments, one immediately after infection and one 5 h later.

[b] Fractional inhibitory concentration (FIC) index = PD_{50} of first antibiotic in combination/PD_{50} of first antibiotic alone + PD_{50} of second antibiotic in combination/PD_{50} of second antibiotic alone = index ≤0.5 indicative of synergy.

[c] PD_{50} value equals the amount of each component in the combination.

cillin when treatment was administered subcutaneously. When ampicillin and amoxicillin were given subcutaneously and FL1039 orally, or FL1060 was given subcutaneously and either of the two antibiotics orally, synergy was seen with amoxicillin but not with ampicillin. In the case of *K. pneumoniae* PM, synergy was noted when ampicillin was given subcutaneously and FL1039 orally, when amoxicillin and FL1039 were both given orally, and when amoxicillin was given orally and FL1060 was administered subcutaneously.

No synergy was demonstrated with combinations of amoxicillin and ampicillin when both agents were given either subcutaneously or orally.

The parenteral amidinopenicillin FL1060 was also quite effective as a synergistic agent when combined with cefazolin and tested against the two *E. coli* strains, *P. mirabilis*, *P. vulgaris* 100, *K. pneumoniae*, *E. cloacae*, and *H. influenzae* (Table 3). In the case of *P. vulgaris* ATCC 6380, synergy was not observed with the combination of cefazolin and FL1060.

The results of subcutaneous treatment with a 1:1 combination of FL1060 and carbenicillin and with the single agents are shown in Table 4. Definite synergy was observed with the combination in the *P. vulgaris* and *K. pneumoniae* infections but not in the *P. aeruginosa* and *E. coli* infections.

When FL1060 was combined with benzylpenicillin and tested against three gram-positive bacteria, synergy was only observed in the *S. aureus* infection (Table 5). FL1060 alone, as previously mentioned, did not appear to be particularly active in these three gram-positive bacterial infections (FL1060 PD_{50} range of 36 to 74 mg/kg versus benzylpenicillin PD_{50} range of 0.33 to 6.0 mg/kg).

No synergy was demonstrated between FL1060 and erythromycin or oxytetracycline in selected gram-negative or the three gram-positive bacterial infections mentioned above.

TABLE 2. *Effect of administering ampicillin or amoxicillin combined in a 1:1 ratio with the oral FL1039 and parenteral FL1060 6β-amidinopenicillins or with each other by the subcutaneous, oral, or subcutaneous and oral routes against gram-negative bacterial infections in mice*

Organism	Route of administration[a]	FIC index[b]				
		Ampicillin +		Amoxicillin +		Ampicillin + amoxicillin
		FL1039	FL1060	FL1039	FL1060	
E. coli 503-455	po/po	0.21		0.23		>1
	sc/po	0.08		≤0.15		
	po/sc		0.24		0.08	
P. mirabilis 2	po/po	0.26		0.20		>1
	sc/po	>1		≤0.47		
	sc/sc		0.32		0.24	>1
	po/sc		0.69		0.43	
P. vulgaris 100	po/po	0.30		≤0.39		>1
	sc/po	≤0.16		0.33		
	sc/sc		0.09		0.20	>1
	po/sc		0.14		0.11	
K. pneumoniae A	po/po	≤0.12		≤0.24		0.78
	sc/po	≤0.04		≤0.31		
	sc/sc		≤0.07		≤0.28	>1
	po/sc		0.20		0.22	
K. pneumoniae 1	po/po	0.35		≤0.35		>1
	sc/po	0.09		0.12		
	sc/sc		0.10		0.11	0.91
	po/sc		0.20		≤0.16	
K. pneumoniae PM	po/po	≤0.80		≤0.38		>1
	sc/po	0.30		0.59		
	sc/sc		≤0.92		≤0.96	>1
	po/sc		≤0.70		≤0.51	
E. cloacae 503-964	po/po	0.12		0.20		0.96
	sc/po	≤0.15		0.17		
	sc/sc		0.23		0.27	0.96
	po/sc		0.15		0.05	

[a] Two treatments, one immediately after infection and one 5 h later. po, Per os; sc, subcutaneously.
[b] See Table 1 for definition of FIC index.

TABLE 3. *Effect of administering cefazolin and FL1060 alone and combined in a 1:1 ratio subcutaneously against gram-negative bacterial infections in mice*

Organism	PD_{50} (mg/kg)			FIC index[a]
	Cefazolin	FL1060	Cefazolin + FL1060	
E. coli 257[b]	1.4	2.3	0.13	0.15
E. coli 5152[b]	27	>250	6.8	≤0.28
P. mirabilis 2[c]	9.6	166	3.5	0.39
P. vulgaris 100[c]	132	87	8.1	0.15
P. vulgaris ATCC 6380[c]	22	<0.9	0.58	≥0.67
K. pneumoniae A[c]	25	415	3.4	0.14
E. cloacae 503-964[c]	10	139	1.1	0.12
H. influenzae 5[c]	8.7	15	0.75	0.14

[a] See Table 1 for definition of FIC index.
[b] One treatment immediately after infection.
[c] Two treatments, one immediately after infection and one 5 h later.

Additional experiments were carried out to determine whether FL1060 would act synergistically with the aminoglycoside antibiotic gentamicin in representative gram-negative bacterial infections. No synergy was observed except in the case of *E. coli* 5152 (Table 6).

DISCUSSION

The difficulties involved in finding entirely new classes of antibiotics are well known. Failure to find antibiotics different in structure and mechanism of action from those currently available has resulted in a search for alternative means of treatment, such as combination therapy with two agents which would act synergistically. The most notable example of a successful synergistic combination has been that of a sulfonamide, sulfamethoxazole, with the 2,4-diamino-pyrimidine, trimethoprim. This combination is synergistic against a broad range of microorganisms (1).

The rationale for combining a sulfonamide

TABLE 4. *Effect of administering carbenicillin and FL1060 alone and combined in a 1:1 ratio subcutaneously against gram-negative bacterial infections in mice*

Organism	PD_{50} (mg/kg)[a]			FIC index[b]
	Carbenicillin	FL1060	Carbenicillin + FL1060	
P. vulgaris 100	193[c]	53	7.2	0.17
K. pneumoniae 1	276[d]	53	5.2	0.12
P. aeruginosa 503-820	229[c]	161	>250	>1.0
E. coli 5152	>500[d]	>500	>500	>1.0

[a] Two treatments, one immediately after infection and one 5 h later.
[b] See Table 1 for definition of FIC index.
[c] Indanyl carbenicillin sodium salt.
[d] Carbenicillin disodium salt.

TABLE 5. *Effect of administering benzylpenicillin and FL1060 alone and combined in a 1:1 ratio subcutaneously[a] against gram-positive bacterial infections in mice*

Organism	PD_{50} (mg/kg)			FIC index[b]
	Benzylpenicillin	FL1060	Benzylpenicillin + FL1060	
S. aureus Smith	6.0	74	1.1	0.2
S. pyogenes 4	0.33	36	0.78	>1.0
S. pneumoniae 6301	3.5	53	6.6	>1.0

[a] One treatment immediately after infection.
[b] See Table 1 for definition of FIC index.

TABLE 6. *Effect of administering gentamicin and FL1060 alone and combined in a 1:1 ratio subcutaneously[a] against gram-negative bacterial infections in mice*

Organism	PD_{50} (mg/kg)			FIC index[b]
	Gentamicin	FL1060	Gentamicin + FL1060	
E. coli 5152	7.4	>500	4.0	≤0.55
P. vulgaris ATCC 6380	5.0	7.9	9.2	>1.0
K. pneumoniae A	0.82	>500	0.56	≤0.68
E. cloacae 503-964	0.52	38	0.66	>1.0
P. aeruginosa 503-820	4.6	>500	7.3	>1.0

[a] Two treatments, one immediately after infection and one 5 h later.
[b] See Table 1 for definition of FIC index.

with trimethoprim was based on the fact that these agents blocked different steps on the same pathway in the bacterial cell. Thus, through a double blockade, the two agents would be able to act in a synergistic fashion. Extensive experimental and clinical data have confirmed that this combination was in fact synergistic (1).

The 6β-amidinopenicillins have been shown to exert their antibacterial activity primarily against gram-negative bacteria at a point different from that of the other known penicillins (3, 6). Thus, we have a situation analogous to that shown for the sulfonamide-trimethoprim combination; two agents acting at different points on a common synthetic pathway.

From the data presented, it is apparent that the amidinopenicillins, when combined with 6-aminopenicillanic acid and 7-aminocephalosporanic acid derivatives, are in fact capable of exerting in vivo a synergistic effect against a number of gram-negative infections.

Thus, the data presented in these animal studies confirm and extend the in vitro results, which suggested that the amidinopenicillins may be clinically useful, perhaps as single agents but more interestingly in conjunction with other penicillins and cephalosporins.

LITERATURE CITED

1. **Finland, M., and E. H. Kass (ed.).** 1973. Trimethoprim-sulfamethoxazole. J. Infect. Dis. 128(Suppl.):433-816.
2. **Greenwood, D., and F. O'Grady.** 1973. FL1060—a new beta-lactam antibiotic with novel properties. J. Clin. Pathol. 26:1-6.
3. **Greenwood, D., and F. O'Grady.** 1973. The two sites of penicillin action in *Escherichia coli*. J. Infect. Dis. 128:791-794.
4. **James, R., J. Y. Haga, and A. B. Pardee.** 1975. Inhibition of an early event in the cell division cycle of *Escherichia coli* by FL1060, an amidinopenicillanic acid. J. Bacteriol. 122:1283-1292.
5. **Lund, F., and L. Tybring.** 1972. 6β-Amidinopenicillanic acids—a new group of antibiotics. Nature (London) New Biol. 236:135-137.
6. **Matsuhashi, S., T. Kamiryo, P. M. Blumberg, P. Linnett, E. Willoughby, and J. L. Strominger.** 1974. Mechanism of action and development of resistance to a new amidino penicillin. J. Bacteriol. 117:578-587.
7. **Melchior, N. H., J. Blom, L. Tybring, and A. Birch-Andersen.** 1973. Light and electron microscopy of the

early response of *Escherichia coli* to a 6β-amidinopenicillanic acid (FL1060). Acta Pathol. Microbiol. Scand. Sect. B 81:393–407.

8. **Park, J. T., and L. Burman.** 1973. FL1060: a new penicillin with a unique mode of action. Biochem. Biophys. Res. Commun. 51:863–868.
9. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent end points. Am. J. Hyg. 27:493–497.
10. **Roholt, K., B. Nielsen, and E. Kristensen.** 1975. Pharmacokinetic studies with mecillinam and pivmecillinam. Chemotherapy (Basel) 21:146–166.
11. **Tybring, L., and N. H. Melchior.** 1975. Mecillinam (FL1060), a 6β-amidinopenicillanic acid derivative: bactericidal action and synergy in vitro. Antimicrob. Agents Chemother. 8:271–276.