IN VITRO SYNERGISM OF FR-31564, A NEW PHOSPHONIC ACID ANTIBIOTIC

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(Received for publication February 25, 1981)

Against most test strains of Gram-negative bacilli, the *in vitro* effect of FR-31564 together with β-lactam antibiotics or trimethoprim was strongly synergistic; with tetracycline and nalidixic acid the effect was additive; and with gentamicin and sulfamethoxazole the effect was additive or antagonistic.

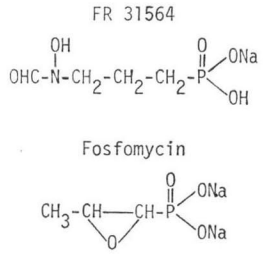
FR-31564 was markedly synergistic with β-lactam antibiotics against β-lactam antibiotic-resistant Gram-negative bacilli such as *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *E. cloacae*, *Citrobacter freundii* and *Serratia marcescens*.

The combination of FR-31564 with β-lactam antibiotics effected a reduction of MICs against most of the test strains to clinically achievable concentrations in human serum.

FR-31564, a new phosphonic acid antibiotic[1], is active against most Gram-negative bacteria including *Pseudomonas aeruginosa* but is not active against *Serratia marcescens* and other glucose-nonfermentative Gram-negative rods. In our previous papers, we reported the *in vitro* and *in vivo* antibacterial activities[2], bacterial resistance and membrane permeability to FR-31564[3]. Recently fosfomycin[4~7] and alafosfalin[8,9], drugs similar in chemical structure to FR-31564 (Fig. 1), have been found to be synergistic with various kinds of antibiotics.

In this paper we report the results of tests for synergism between FR-31564 and other antibiotics against various organisms.

Fig. 1. Chemical structure of FR-31564 and fosfomycin.



Materials and Methods

Antibiotics

FR-31564 and fosfomycin were provided by the Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan; ampicillin and carbenicillin by Beecham Pharmaceuticals, Betchworth, England; cefazolin by Fujisawa Pharmaceutical Co., Ltd.; cephalexin by Eli Lilly & Co., Indianapolis, U.S.A.; tetracycline by Pfizer Inc., New York, U.S.A.; nalidixic acid by Winthrop Laboratories, New York, U.S.A.; trimethoprim and sulfamethoxazole by Burroughs Wellcome Co., North Carolina, U.S.A.; and gentamicin by Schering Corporation, New Jersey, U.S.A.

Bacteria

Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes, E. cloacae, Serratia marcescens, Citrobacter freundii, Proteus vulgaris, Pseudomonas aeruginosa, and *Staphylococcus aureus* were clinically isolated from patients in several hospitals in Japan.

Antimicrobial Activity and Synergism

The antimicrobial activity of the test antibiotics was determined by the agar dilution method.

Thousand-fold dilutions of overnight bacterial cultures in nutrient broth (Eiken) were inoculated with a multiple inoculator onto nutrient agar (Difco) containing graded concentrations of the test drugs. The minimum inhibitory concentrations (MICs) were determined after incubation at 37°C for 18~20 hours. For sulfamethoxazole MICs, MUELLER-HINTON agar (Eiken) and MUELLER-HINTON broth (Difco) were used as the test medium and preculture medium, respectively. Synergism was studied as follows: The combination ratios of FR-31564 to the test antibiotics were 4: 1, 1: 1 and 1: 4 except the ratios between FR-31564 and gentamicin which were 10: 1, 4: 1 and 1: 1. The fractional inhibitory concentration (FIC) index was calculated by the method of WEINSTEIN *et al.*[10] The effect was defined as synergistic when the FIC index was equal to or less than 0.5, as additive when 0.5~1.0 and as antagonistic when more than 1.0.

$$\text{FIC index} = \frac{\text{MIC of Drug A in comb.}}{\text{MIC of Drug A alone}} + \frac{\text{MIC of Drug B in comb.}}{\text{MIC of Drug B alone}}$$

The existence of synergism was evaluated from the smallest FIC value of the 3 combination ratios. Geometric mean MICs were calculated from the values obtained with the ratio 1: 4 of FR-31564 to the test antibiotics.

Bactericidal Effect

Nutrient broths containing concentrations of 40 μg/ml (1/5 the MIC) of FR-31564 alone and in combination with 1, 5, 25 and 125 μg/ml of β-lactam antibiotics were inoculated with 0.5% of an overnight culture of *E. aerogenes* No. 7 to obtain about 10^6 colony forming units (c.f.u.)/ml, and incubated at 37°C for 3 hours. Viable cell counts in the culture fluids were determined by conventional plating techniques.

Results

1. FIC Index

The MICs of FR-31564 alone and in combination with the other test antibiotics were determined against test organisms resistant to FR-31564. The synergistic effect between FR-31564 and the other antibiotics was evaluated by FIC index.

Table 1 shows that FR-31564 was synergistic with cefazolin against all strains of *E. aerogenes, E. cloacae, C. freundii* and *S. aureus*, and against 80, 60, and 40% of the test strains of *S. marcescens, P. vulgaris* and *K. pneumoniae* respectively. However, FR-31564 was additive with cefazolin against 83% of *E. coli* strains. The combination of FR-31564 with cephalexin showed synergism against all strains of *S. marcescens* and *C. freundii*, and against 80% of each of the strains of *K. pneumoniae, E. aerogenes* and *P. vulgaris* tested. On the other hand, the frequency of synergism between FR-31564 and cephalexin was low against *P. aeruginosa* (50%), *E. cloacae* (20%) and *E. coli* (17%). The combination of FR-31564 with cephalexin exerted scarcely any synergistic effect against strains of *S. aureus*.

FR-31564 was synergistic with ampicillin and carbenicillin against many of the test organisms but was not synergistic with carbenicillin against *S. aureus*. Synergism was seen less frequently when FR-31564 was combined with nalidixic acid and tetracycline than when combined with the above β-lactam antibiotics. FR-31564 was significantly synergistic with trimethoprim against all organisms except *P. vulgaris* and *P. aeruginosa*. FR-31564 was slightly synergistic with sulfamethoxazole and gentamicin against the organisms tested.

These results indicate that the β-lactam antibiotics and trimethoprim were suitable partners for synergistic combinations with FR-31564 and gave synergistically effective combinations against *K. pneumoniae, E. aerogenes, E. cloacae, S. marcescens* and *C. freundii*.

Table 1. Synergism of FR-31564 with other antibiotics against clinical isolates.

Antibiotic	Organism (No. of strains)	Percent of synergism (%)		
		Synergy	Addition	Antagonism
Cefazolin	*E. coli* (6)		83	17
	K. pneumoniae (5)	40	40	20
	E. aerogenes (5)	100		
	E. cloacae (5)	100		
	S. marcescens (5)	80		20
	C. freundii (5)	100		
	P. vulgaris (5)	60	40	
	S. aureus (5)	100		
Cephalexin	*E. coli*	17	83	
	K. pneumoniae	80	20	
	E. aerogenes	80	20	
	E. cloacae	20	80	
	S. marcescens	100		
	C. freundii	100		
	P. vulgaris	80		20
	P. aeruginosa (6)	50	33	17
	S. aureus		20	80
Ampicillin	*E. coli*	50	50	
	K. pneumoniae	100		
	E. aerogenes	100		
	E. cloacae	100		
	S. marcescens	40	40	20
	C. freundii	100		
	P. vulgaris	80		20
	S. aureus	60		40
Carbenicillin	*E. coli*	83	17	
	K. pneumoniae	100		
	E. aerogenes	100		
	E. cloacae	100		
	S. marcescens	20	60	20
	C. freundii	100		
	P. vulgaris	40	40	20
	P. aeruginosa		83	17
	S. aureus			100
Nalidixic acid	*E. coli*		80	20
	K. pneumoniae	20	80	
	E. aerogenes	40	40	20
	E. cloacae	80	20	
	S. marcescens	40	20	40
	C. freundii	20	60	20
	P. vulgaris		20	80
	S. aureus		40	60

Table 1. (Continued)

Antibiotic	Organism (No. of strains)	Percent of synergism (%)		
		Synergy	Addition	Antagonism
Tetracycline	*E. coli*	50	34	16
	K. pneumoniae	40	20	40
	E. aerogenes	20	20	60
	E. cloacae	40	20	40
	S. marcescens	20	80	
	C. freundii	20	60	20
	P. vulgaris		20	80
	P. aeruginosa	17	50	33
Trimethoprim	*E. coli*	83	17	
	K. pneumoniae	60	40	
	E. aerogenes	80	20	
	E. cloacae	100		
	S. marcescens	80	20	
	C. freundii	80	20	
	P. vulgaris		60	40
	P. aeruginosa	17	83	
Sulfamethoxazole	*E. coli*		67	33
	K. pneumoniae	40	40	20
	E. aerogenes		60	40
	E. cloacae	60	20	20
	S. marcescens	20	40	40
	C. freundii	20	40	40
	P. vulgaris		60	40
	P. aeruginosa	33		67
Gentamicin	*E. coli*		17	83
	K. pneumoniae		20	80
	E. aerogenes	20	40	40
	E. cloacae	20	60	20
	S. marcescens		80	20
	C. freundii	40	20	40
	P. vulgaris		80	20
	P. aeruginosa		33	67

Synergy; FIC index$\leqq$0.5.
Addition; 0.5<FIC index$\leqq$1.
Antagonism; FIC index>1.

2. MIC Values

Geometric mean MICs of FR-31564 and β-lactam antibiotics, alone and in combination (ratio; 1 : 4) were compared to evaluate the antibacterial efficacy of these combination's.

Table 2 shows the mean MICs of FR-31564 alone and in combination with cefazolin, cephalexin, ampicillin or carbenicillin against 5 or 6 strains of each of the test organisms. MICs against *S. aureus*, which is highly susceptible to β-lactam antibiotics but not to FR-31564, were either slightly reduced or unaffected by the combination of two drugs.

Table 2. Mean MICs of FR-31564 alone and in combination with β-lactams against FR-31564-resistant strains.

Organism (No. of strains)		Mean MICs (μg/ml)				
		Alone	Combination			
			Cefazolin	Cephalexin	Ampicillin	Carbenicillin
S. aureus (5)	—	—	(0.30)	(2.06)	(0.34)	(0.90)
	FR-31564	(459)	0.20	2.24	0.38	2.26
E. coli (6)	—	—	(1.75)	(6.25)	(14.0)	(70.7)
	FR-31564	(35.3)	1.74	6.96	6.19	12.4
K. pneumoniae (5)	—	—	(2.37)	(12.5)	(16.5)	(132)
	FR-31564	(50.0)	1.29	5.92	5.91	11.9
E. cloacae (5)	—	—	(87.0)	(57.4)	(65.9)	(57.4)
	FR-31564	(7.17)	2.24	5.91	1.70	1.70
E. aerogenes (5)	—	—	(200)	(528)	(174)	(50.0)
	FR-31564	(75.8)	11.8	27.2	15.6	10.3
S. marcescens (5)	—	—	(606)	(382)	(303)	(348)
	FR-31564	(75.8)	47.3	17.9	47.3	71.7
C. freundii (5)	—	—	(174)	(263)	(99.9)	(43.5)
	FR-31564	(25.0)	7.81	7.81	3.39	6.80
P. vulgaris (5)	—	—	(14.4)	(43.5)	(33.0)	(3.60)
	FR-31564	(37.9)	7.81	10.3	6.80	3.40

( ); Mean MICs (μg/ml) of antibiotic alone.
Ratio; FR-31564 - β-lactam, 1 : 4.

Table 3. Comparison of mean MICs of FR-31564 and fosfomycin in combination with β-lactams against clinical isolates.

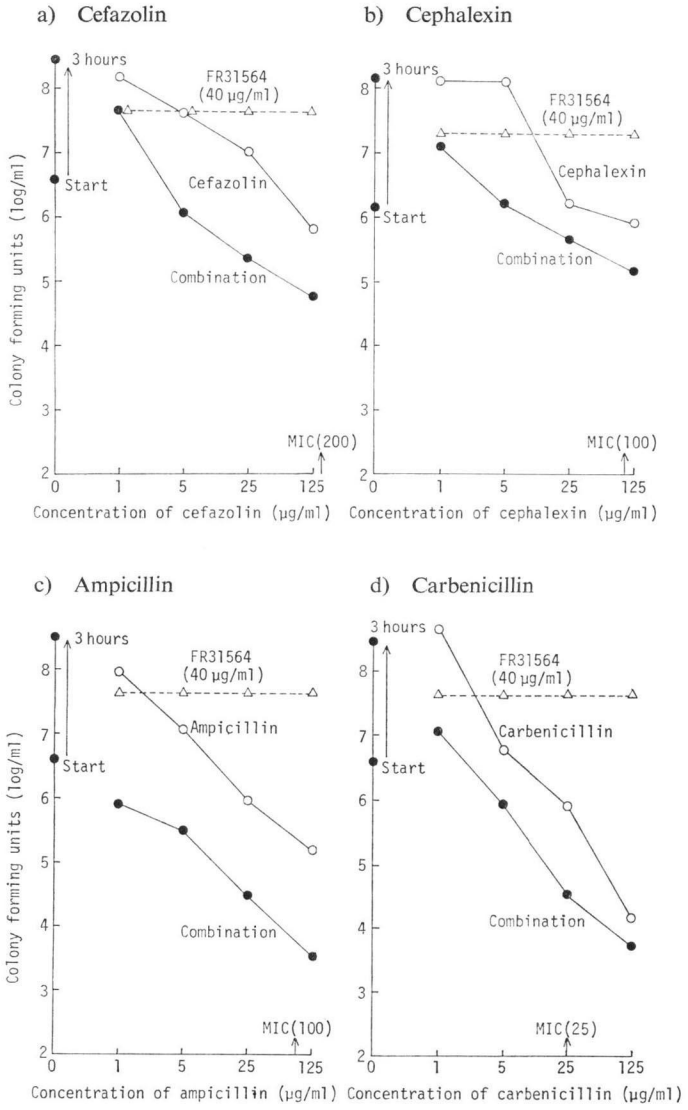
Organism (No. of strains)		Mean MICs (μg/ml)				
		Alone	Combination			
			Cefazolin	Cephalexin	Ampicillin	Carbenicillin
E. coli (21)	—	—	(2.25)	(11.3)	(28.5)	(110)
	FR-31564	(9.92)	2.30	7.81	7.30	16.1
	Fosfomycin	(18.6)	2.46	12.0	9.84	31.2
E. cloacae (21)	—	—	(71.9)	(130)	(42.4)	(38.4)
	FR-31564	(1.85)	1.96	2.81	1.55	2.02
	Fosfomycin	(67.3)	26.5	42.1	20.3	24.8
E. aerogenes (21)	—	—	(51.7)	(149)	(51.7)	(24.2)
	FR-31564	(8.69)	4.92	11.6	5.62	6.00
	Fosfomycin	(13.4)	9.52	17.8	11.2	10.5
C. freundii (21)	—	—	(46.8)	(63.0)	(15.8)	(8.99)
	FR-31564	(2.48)	3.54	5.09	1.89	2.16
	Fosfomycin	(2.32)	3.66	5.43	3.10	3.66

( ); Mean MICs (μg/ml) of antibiotic alone.
Ratio; FR-31564 - β-lactam, 1 : 4.

The reduction of MIC values against organisms highly resistant to the antibiotics tested was generally significant: the degree of synergism was high against *E. cloacae, E. aerogenes, S. marcescens, C. freundii* and *P. vulgaris.*

The synergism of FR-31564 with 4 kinds of β-lactam antibiotics was compared with that of fosfomycin against strains of *E. coli, E. cloacae, E. aerogenes* and *C. freundii* (Table 3). Mean MICs of FR-31564 - β-lactam antibiotic combinations for 21 strains of *E. cloacae* were markedly lower than those

Fig. 2. Bactericidal activity of FR-31564 with β-lactams against *E. aerogenes* No. 7 after 3 hours incubation.



of fosfomycin-β-lactam antibiotic combinations. For the other organisms except *C. freundii*, the mean MICs of FR-31564-β-lactam antibiotic combinations were slightly lower than those of fosfomycin-β-lactam antibiotic combinations but were almost the same for *C. freundii*. These results indicated that concentrations of FR-31564 in combination with β-lactam antibiotics were more effective than those of fosfomycin against some Enterobacteriaceae species.

3. Bactericidal Activity

Killing curves of FR-31564 alone and in combination with β-lactam antibiotics were prepared using FR-31564- and β-lactam antibiotic-resistant *E. aerogenes* strain No. 7. The viable cell counts increased from $1.5 \sim 4.0 \times 10^6$ to $1.7 \sim 3.1 \times 10^8$ c.f.u./ml in the absence of antibiotics. Fig. 2 shows that without exception the viable cell counts were decreased markedly by the combination of FR-31564

and β-lactam antibiotics, although the counts also decreased to some extent in the presence of β-lactam antibiotics alone.

Discussion

Despite the advent of many new antimicrobial agents, serious infections due to Gram-negative bacilli including opportunistic pathogens remain on the increase in compromised hosts[11,12]. Many of the pathogens causing such infections are resistant to antibiotics in common use. We, therefore extensively investigated synergism between antibiotics in order to enhance their therapeutic effect. Synergism increases antibacterial activity; it may expand the antibacterial spectrum and may decrease development of resistant cells. Various antibiotic combinations are widely used in clinical treatment and provide the following mechanisms of synergism: 1) inhibition at two sites of a biochemical pathway (trimethoprim - sulfamethoxazole)[13], 2) prevention of enzymatic hydrolysis by β-lactamase (ampicillin - cloxacillin, β-lactam antibiotics and clavulanic acids or CP45899)[14~16], 3) enhancement of penetration by aminoglycosides to the active site (β-lactam antibiotics and aminoglycosides)[17], and 4) difference of affinity for penicillin binding proteins[18,19].

In addition to the above combinations, phosphonic acid antibiotics such as fosfomycin and alafosfalin have recently been reported to act synergistically with various kinds of antibiotics, especially with β-lactam antibiotics. FR-31564 also was markedly synergistic with β-lactam antibiotics and trimethoprim. The extent of *in vitro* synergism is generally estimated on the basis of the FIC-index. The MICs against most of the test strains of various organisms can be reduced to concentrations achievable in human serum by synergistic combinations. Although most of the strains were highly resistant to individual antibiotics, the combination of FR-31564 with β-lactam antibiotics gave often a high efficiency. The combinations of FR-31564 and β-lactam antibiotics were generally more active *in vitro* than those of fosfomycin with β-lactam antibiotics. The high ability of FR-31564 to penetrate the bacterial membrane[3] may contribute to its synergistic effect when used with β-lactam antibiotics, although the mechanism of synergism between phosphonic acid antibiotics and β-lactam antibiotics has not been clarified. Recent reports have shown that resistant mutants appear at high frequency in the presence of fosfomycin and the same phenomenon has also been observed for FR-31564.

Since there is a possibility that resistant mutants may arise by the clinical use of FR-31564 alone, the combination of FR-31564 and β-lactam antibiotics should be considered in order to avoid this potential clinical problem.

References

1) Okuhara, M.; Y. Kuroda, T. Goto, M. Okamoto, H. Terano, M. Kohsaka, H. Aoki & H. Imanaka: Studies on new phosphonic acid antibiotics. III. Isolation and characterization of FR-31564, FR-32863 and FR-33289. J. Antibiotics 33: 24~28, 1980
2) Mine, Y.; T. Kamimura, S. Nonoyama, M. Nishida, S. Goto & S. Kuwahara: *In vitro* and *in vivo* antibacterial activities of FR-31564, a new phosphonic acid antibiotic. J. Antibiotics 33: 36~43, 1980
3) Kojo, H.; Y. Shigi & M. Nishida: FR-31564, a new phosphonic acid antibiotic: Bacterial resistance and membrane permeability. J. Antibiotics 33: 44~48, 1980
4) Borowski, J. & H. Linda: Combined action of fosfomycin with β-lactam and aminoglycoside antibiotics. Chemotherapy 23: 82~85, 1977
5) Daza, R.; M. Moreno-Lopez & D. Damaso: Interaction of fosfomycin with other antibiotics. Chemotherapy 23: 86~92, 1977
6) Olay, T.; A. Rodriguez, L. E. Oliver, M. V. Vicente & M. C. R. Quecedo: Interaction of fosfomycin with other antibacterial agents: *in vitro* and *in vivo* studies. J. Antimicr. Chemoth. 4: 569~576, 1978
7) Perea, E. J.; M. A. Torres & M. V. Borobio: Synergism of fosfomycin-ampicillin and fosfomycin-chloramphenicol against *Salmonella* and *Shigella*. Antimicr. Agents & Chemoth. 18: 705~709, 1978
8) Allen, J.G.; F. R. Atherton, M. J. Hall, C. H. Hassall, S. W. Holmes, R. W. Lambert, L. J. Nisbert & P. S. Ringrose: Phosphonopeptides as antibacterial agents; Alaphosphin and related phosphonopeptides. Antimicr. Agents & Chemoth. 15: 684~695, 1979

9) MARUYAMA, H. B.; M. ARISAWA & T. SAWADA: Alafosfalin, a new inhibitor of cell wall biosynthesis: *In vitro* activity against urinary isolates in Japan and potentiation with β-lactams. Antimicr. Agents & Chemoth. 16: 444~451, 1979
10) WEINSTEIN, R. J.; L. S. YOUNG & W. L. HEWITT: Comparison of methods for assessing *in vitro* antibiotic synergism against *Pseudomonas* and *Serratia*. J. Lab. Clin. Med. 86: 853~862, 1975
11) FASS, R. J.: Treatment of mixed bacterial infections with clindamycin and gentamicin. J. Infect. Dis. 135: 74~79, 1977
12) KLASTERSKY, J.; R. CAPPEL & D. DANEAU: Clinical significance of *in vitro* synergism between antibiotics in Gram-negative infections. Antimicr. Agents & Chemoth. 2: 470~475, 1972
13) YOKOTA, T.: Clinical evaluation of assay methods for antimicrobial activities of sulfamethoxazole and trimethoprim with a report on the potentiation of drug activities by the combination against clinically isolated bacterial strains. Chemotherapy 21: 117~126, 1973
14) GREENWOOD, D.; F. O'GRADY & P. RAKER: An *in vitro* evaluation of clavulanic acid, a potent broad-spectrum β-lactamase inhibitor. J. Antimicr. Chemoth. 5: 539~547, 1979
15) NISHIDA, M.; Y. MINE & S. KUWAHARA: Synergistic activity of ampicillin and cloxacillin. Protective effect of cloxacillin on enzymatic degradation of ampicillin by penicillinase, and therapeutic activity of mixtures of ampicillin and cloxacillin. J. Antibiotics 22: 144~150, 1969
16) DUMON, L.; P. ADRIAENS, J. ANNE & H. EYSSEN: Effect of clavulanic acid on the minimum inhibitory concentration of benzylpenicillin, ampicillin, carbenicillin, or cephalothin against clinical isolates resistant to beta-lactam antibiotics. Antimicr. Agents & Chemoth. 15: 315~317, 1979
17) MASUDA, G.; T. YAJIMA, K. NAKAMURA, T. YANAGISHITA & E. YAMAZAKI: Effect of inhibitory and sub-inhibitory concentrations of tobramycin on bacteriostatic and bactericidal activities of β-lactam antibiotics. Jap. J. Antibiotics 32: 1189~1195, 1979
18) GRAY, J.: Synergy and mecillinam. J. Antimicr. Chemoth. 3: 531~533, 1977
19) KERRY, D. W.; J. M. T. HAMILTON-MILLER & W. BRUMFITT: Laboratory studies on mecillinam: Activity alone and combined with cephradine. J. Antimicr. Chemoth. 3: 53~58, 1977