FR-31564, A NEW PHOSPHONIC ACID ANTIBIOTIC: BACTERIAL RESISTANCE AND MEMBRANE PERMEABILITY

HITOSHI KOJO, YASUTAKA SHIGI and MINORU NISHIDA*

Research Laboratories, Fujisawa Pharmaceutical Co., Ltd.,
Osaka, Japan

(Received for publication September 6, 1979)

Mutants which acquired resistance to FR-31564 were also resistant to fosfomycin and *vice versa*. Some exceptions to cross-resistance were found among clinical isolates of certain species. FR-31564 was found to be incorporated into bacterial cells more efficiently than fosfomycin although the extent of incorporation varied among species. In particular, the uptake rate of FR-31564 by a strain of *Pseudomonas aeruginosa* was ten times that of fosfomycin. The uptake rate of FR-31564 by both FR-31564- and fosfomycin-resistant mutants was less than one-tenth of that by the parent strain. FR-31564 was scarcely inactivated in the culture broths of FR-31564-resistant strains.

All of the FR-31564-resistant mutants of *P. aeruginosa* came under the classification of strains lacking an L-α-glycerophosphate transport system.

Extensive studies performed on the permeability of the bacterial membrane to fosfomycin[1~6] have led to the conclusion that fosfomycin is incorporated into bacterial cells by active transport systems and that bacterial resistance to fosfomycin results from the loss of these systems[1].

FR-31564, a new antibiotic chemically related to fosfomycin[7] exhibits cross-resistance with fosfomycin, a finding that prompted us to investigate bacterial membrane permeability to FR-31564 and to fosfomycin.

In this paper, some similarities and differences in the membrane permeabilities of FR-31564 and fosfomycin are described.

Materials and Methods

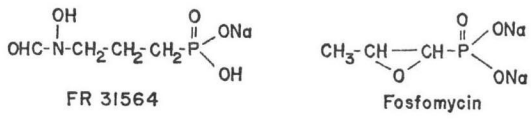
1. Antibiotics and reagents

FR-31564 and fosfomycin were prepared by the Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. Glucose-6-phosphate, L-α-glycerophosphate, and cyclic adenosine monophosphate (cAMP) were purchased from Sigma Chemical Co. All other chemicals were of reagent grade.

2. Bacterial strains

Escherichia coli K 12 HfrH was supplied by Dr. T. YOKOTA of Juntendo University, *Pseudomonas aeruginosa* 5-70 by Dr. E. YABUUCHI of Kansai Medical University, and *Proteus* sp. MB 838 by Dr. S. GOTO of Toho University. *Enterobacter cloacae* ATCC 29893 and all of the other strains used in this study were isolated from clinical sources.

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



* Full address: Dr. M. NISHIDA, Director of Chemotherapy Research Division, Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 1-6, 2-Chome, Kashima, Yodogawa-ku, Osaka 532, Japan

3. Antibiotic susceptibility tests

Antibiotic susceptibility was determined by the agar dilution method. Millesimal dilutions of overnight bacterial cultures in nutrient broth (Eiken Chemical Co., Tokyo) were spot-inoculated with a multiple inoculator onto nutrient agar (Difco Laboratories) containing graded concentrations of the test drugs. The minimum inhibitory concentration (MIC) was determined after incubation at 37°C for 20 hours.

4. Isolation of FR-31564- and fosfomycin-resistant mutants

The following alternative methods were used for the isolation of the resistant mutants.

(a) When the *in vitro* antibiotic susceptibility was determined by the agar dilution method, a spot of approximately 10^3 parent cells was inoculated on an agar plate containing half the minimum inhibitory concentration of the antibiotic. After incubation, the colonies on the plate which were approximately the same in size as the parent colonies were few (<10). These colonies were picked up and tested for the antibiotic susceptibility. Cells inhibited only at more than 4 times the concentrations for the parent strains were regarded as resistant mutants.

(b) An overnight culture of the test strain in nutrient broth was spread on the nutrient agar containing 400 μg/ml of the test antibiotic to make the population of $10^5 \sim 10^7$ cells per agar plate. After incubation at 37°C for 20 hours, eighty colonies grown on the plate containing the appropriate number of colonies ($10^2 \sim 10^3$ cells per plate) were picked up randomly as resistant mutants and used for classification purposes.

5. Antibiotic assays

The concentrations of FR-31564 and fosfomycin in samples were determined by microbiological assays.

(a) Assay for FR-31564: Assay cultures of *E. cloacae* ATCC 29893 were grown overnight in nutrient broth (Eiken Chemicals) and inoculated into nutrient agar (Difco) at a concentration of 0.5%. The agar was thoroughly mixed with the bacterial culture, poured into Petri dishes (80 mm) and allowed to harden. Thick paper discs (8 mm) immersed in the antibiotic solutions were placed on the above plates. After incubation for 20 hours at 37°C, the diameters of the inhibitory zones around the discs were measured. The antibiotic concentration in the sample was determined by a calibration curve prepared using known amounts of antibiotic and the diameter of the resulting inhibition zones.

(b) Assay for fosfomycin: The assay method for fosfomycin was the same as that for FR-31564 except that *Proteus* sp. MB 838 was used as the assay organism and that the inoculum size was 1.0%.

6. Measurement of intracellular accumulation of FR-31564 and fosfomycin

The reaction mixture used for uptake of the antibiotics had a final volume of 5.0 ml: 0.5 ml of antibiotic (10 mg/ml), 1.0 ml of cell suspension (approximately 10^{11} cells/ml) and 3.5 ml of nutrient broth (Difco). The cell suspension was prepared as follows: Cells grown overnight at 37°C in nutrient broth were harvested by centrifugation, washed once and resuspended in the same broth (one-hundredth volume of the original culture). After incubation for 20 minutes at 37°C, the cells were washed twice with 50 mM tris buffer (pH 7.0) by centrifugation at 8,000 g for 5 minutes to remove the antibiotic. The cells were resuspended in the same buffer and sonicated at maximum power for 10 minutes with cooling. Antibiotic concentration in the sonicate was determined by microbiological assay. Uptake of the antibiotic was expressed as ng of incorporated antibiotic per mg of cellular protein. Protein was determined according to the method of LOWRY *et al.* using bovine serum albumin as the standard[8].

7. Test for stability of FR-31564 in culture fluids of resistant mutants

FR-31564-resistant mutants of various species were grown overnight at 37°C in heart infusion broth. One ml of the overnight culture was inoculated into 3.5 ml of the broth and incubated at 37°C for 2 hours with shaking. Then, 0.5 ml of FR-31564 solution (200 μg/ml) was added to the incubation mixture. After further incubation at 37°C for 3 and 6 hours, the reaction was stopped by the addition of an equal volume of ethanol. Any precipitate formed was removed by centrifugation. Residual content of FR-31564 in the resultant supernatant was determined by microbiological assay.

8. Examination of carbohydrate utilization of FR-31564-resistant mutants

Test strains grown on the master plates were replicated on the minimal agar medium modified from E medium of VOGEL and BONNER[9]. Minimal medium without carbon sources contained (per liter of distilled water) $(NH_4)_2SO_4$, 1 g; KH_2PO_4, 10 g; $MgSO_2 \cdot 7H_2O$, 0.1 g; and agar, 15 g. pH was adjusted to 7.0 with 10 M KOH.

Carbohydrates were sterilized independently and added to the medium to give a final concentration of 1 g per liter. Carbohydrates used were as follows: L-α-glycerophosphate, glucose-6-phosphate, glycerol, L-α-glycerophosphate plus cAMP and glycerol plus cAMP. cAMP was supplemented to give a final concentration of 500 μg/ml.

Results and Discussion

1. Cross-resistance between FR-31564 and Fosfomycin

Most of the mutants which acquired resistance to FR-31564 were also resistant to fosfomycin. The converse was also established (Table 1). However, some exceptions to cross-resistance between FR-31564 and fosfomycin were found among clinical isolates of some species (Table 2).

Table 1. Cross-resistance against FR-31564 and fosfomycin, in highly resistant mutants.

Organism	MIC (μg/ml)			
	Mutant		Parent	
	FR-31564	Fosfomycin	FR-31564	Fosfomycin
P. aeruginosa 25*	>800	>800	50	50
E. coli 54*	400	200	100	50
K. pneumoniae 74*	800	>800	50	50
E. aerogenes 35*	>800	>800	100	400
S. marcescens 35*	>800	800	12.5	3.13
P. vulgaris 49*	200	100	6.25	1.56
P. rettgeri 34*	400	>800	3.13	25
P. aeruginosa 25**	>800	>800	50	50
E. coli 54**	400	200	100	50

*: FR-31564-resistant mutant.
**: Fosfomycin-resistant mutant.
MIC: nutrient agar (Difco), 37°C, 20 hours. 10^{-3} dilution (inoculum).

2. Intracellular Incorporation of FR-31564 and Mechanism of FR-31564-resistance

The uptake of FR-31564 by strains of various species was compared with that of fosfomycin. FR-31564 was found to be incorporated into bacterial cells more efficiently than fosfomycin although the degree of difference in incorporation varied among species (Table 3). Of

Table 2. Differences in susceptibility of some clinical isolates to FR-31564 and fosfomycin.

Organism	Mean MIC (μg/ml)		MIC of selected strains (μg/ml)		
	FR-31564	Fosfomycin	Strain	FR-31564	Fosfomycin
P. vulgaris (50)	2.8	1.9	No. 28	3.13	12.5
P. mirabilis (50)	5.2	1.2	No. 66	6.25	25
			No. 80	25	3.13
K. pneumoniae (100)	58	76	No. 265	100	>400
E. aerogenes (50)	25	37	No. 14	100	50
			No. 32	12.5	200
E. cloacae (100)	2.1	87	No. 60	12.5	1.56

MIC: nutrient agar, 37°C, 18~20 hours. 10^{-3} dilution (inoculum)
Figures in parentheses indicate number of strains tested.

Table 3. Bacterial membrane permeability to FR-31564 and fosfomycin.

Strain	Antibiotic	MIC (μg/ml)	Uptake (μg/mg protein)		Ratio FR-31564/ fosfomycin
			0 min.	20 min.	
E. coli K12 HfrH	FR-31564	25	0.91	6.57	1.78
	Fosfomycin	6.25	<0.10	3.70	
S. marcescens No. 4	FR-31564	25	0.30	3.03	1.01
	Fosfomycin	3.13	<0.12	3.01	
E. cloacae ATCC 29893	FR-31564	1.56	1.53	8.21	2.61
	Fosfomycin	100	<0.21	3.14	
P. aeruginosa 5–70	FR-31564	6.25	0.48	2.95	42.1
	Fosfomycin	12.5	<0.04	0.07	
S. aureus 209P JC-1	FR-31564	50	<0.52	11.8	3.22
	Fosfomycin	12.5	<0.27	3.67	

Tests were performed as described in the text.

Table 4. Intracellular uptake of FR-31564 by resistant mutants.

Organism	Uptake of FR-31564 (ng/mg protein)	
	0 min.	20 min.
Parent (*P. aeruginosa* 5–70)	54.7	338
FR-31564-resistant mutant	4.8	29.6
Fosfomycin-resistant mutant	5.0	25.1

Tests were performed as described in the text.

Table 5. Stability of FR-31564 in the bacterial culture broths of the antibiotic-resistant mutants.

Strain	MIC (μg/ml)	Residual activity of FR-31564 in % incubation time	
		3 hrs	6 hrs
P. aeruginosa No. 62R*	>800	100	85
P. mirabilis No. 100R	>800	100	84
P. vulgaris No. 49R	25	103	93
P. rettgeri No. 22R	25	92	85
P. inconstans No. 6R	50	97	81
E. coli No. 89R	50	105	95
K. pneumoniae No. 276R	100	103	100
S. marcescens No. 116R	100	94	78
E. cloacae No. 63R	100	94	88
E. aerogenes No. 19R	800	90	87
C. freundii No. 75R	>800	95	93
Control		103	100

* R: resistant mutant

particular note, the uptake of FR-31564 by the test strain of *P. aeruginosa* was 40 times that of fosfomycin. On investigating uptake of FR-31564 by a FR-31564-resistant mutant of *P. aeruginosa* uptake of FR-31564 by the mutant strain was shown to be less than one-tenth that of the parent strain. Similar results were also obtained with a fosfomycin-resistant mutant of *P. aeruginosa* (Table 4).

These results indicate that FR-31564-resistance in bacteria can result from the lack of transport system for FR-31564, used in common for fosfomycin. Meanwhile, inactivation of FR-31564 was ascertained not to contribute to FR-31564-resistance since only slight inactivation was detected after FR-31564 was incubated at 37°C for 6 hours in the culture broths of FR-31564-resistance mutants (Table 5).

3. Active Transport Systems Common to FR-31564 and Fosfomycin

Fosfomycin has been reported to be incorporated into the bacterial cells by two alternative transport systems, namely, L-α-glycerophosphate (genetic designation glp T) and hexose phosphate transport (uhp) systems (Fig. 2)[1].

Accordingly, FR-31564-resistant mutants were examined for their lack of these transport sys-

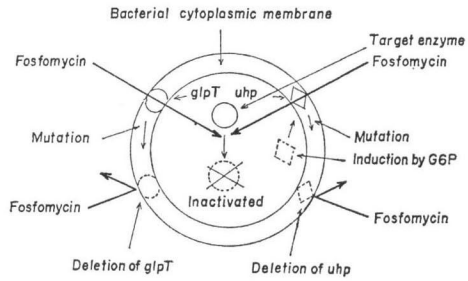
Table 6. Classification of FR-31564-resistant mutants.

Strain	Strain growing on sole carbon source					
	Control (citrate)	αGP	G6P	Glycerol	αGP +cAMP	Glycerol +cAMP
Parent	+*	+	−	+	+	+
FR-31564-resistant mutants (80 strains)	80/80**	0/80	0/80	80/80	0/80	80/80
Fosfomycin-resistant mutants (80 strains)	80/80	2/80	0/80	79/80	2/80	79/80

Test strain used; *P. aeruginosa* 5-70.
Resistant mutants were isolated as described in the text.
Selective concentration of the antibiotic; 400 μg/ml.
Abbreviations: αGP, L-α-glycerophosphate; G6P, glucose-6-phosphate.
*: +, able to grow; −, unable to grow
**: number of strains able to grow per number of strains tested.

tems, by observing their growth on single carbon sources. By this technique the FR-31564-resistant mutants of *P. aeruginosa* were classified as lacking L-α-glycerophosphate transport system (Table 6). (*Pseudomonas* species lack the hexose phosphate transport system). These results indicate that FR-31564 and fosfomycin are transported by the same system. However, the possibility that an additional transport system for FR-31564 might exist cannot be excluded.

Fig. 2. Active transport systems for fosfomycin.
glpT: L-α-glycerophosphate transport system
uhp : hexose phosphate transport system



References

1) Kahan, F. M.; J. S. Kahan, P. J. Cassidy & H. Kropp: The mechanism of action of fosfomycin. Ann. N. Y. Acad. Sci. 235: 364~386, 1974
2) Wu, H. C. & P. S. Venkateswaran: Fosfomycin resistant mutant of *Escherichia coli*. Ann. N. Y. Acad. Sci. 235: 587~592, 1974
3) Tsuruoka, T. & Y. Yamada: Characterization of spontaneous fosfomycin-resistant cells of *Escherichia coli* B *in vitro*. J. Antibiotics 28: 906~911, 1975
4) Cordaro, J. C.; T. Melton, J. P. Stratis, M. Atagrin, C. Gladding, P. E. Hartman & S. Roseman: Fosfomycin resistance: selection method for internal and extended deletions of the phosphoenolpyruvate: sugar phosphotransferase genes of *Salmonella typhimurium*. J. Bacteriol. 128: 785~793, 1976
5) Tsuruoka, T.; A. Miyata & Y. Yamada: Two kinds of mutants defective in multiple carbohydrate utilization isolated from *in vitro* fosfomycin-resistant strains of *Escherichia coli* K-12. J. Antibiotics 31: 192~201, 1978
6) Alper, M. D. & B. N. Ames: Transport of antibiotics and metabolite analogs by systems under cyclic AMP control: positive selection of *Salmonella typhimurium cya* and *crp* mutants. J. Bacteriol. 133: 149~157, 1978
7) Kamiya, T.; K. Hemmi, H. Takeno & M. Hashimoto: Studies on new phosphonic acid-containing antibiotics. II. Synthesis of FR-31564 and related antibiotics. Presentation at the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, Abstract 235, Boston, 1979
8) Lowry, O. H.; N. J. Rosenbrough, A. L. Farr & R. J. Randall: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265~275, 1951
9) Vogel, H. J. & D. M. Bonner: Acetylornithinase of *Escherichia coli*: Partial purification and some properties. J. Biol. Chem. 218: 97~102, 1956