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Mode of Action of the Dual-Action Cephalosporin Ro 23-9424

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Ro 23-9424 is a broad-spectrum antibacterial agent composed of a cephalosporin and a quinolone moiety. Its biological properties were compared with those of its two components and structurally related cephalosporins and quinolones. Like ceftriaxone and cefotaxime but unlike its decomposition product, desacetyl cefotaxime, Ro 23-9424 bound at ≤2 μg/ml to the essential penicillin-binding proteins 1b and 3 of *Escherichia coli* and 1, 2, and 3 of *Staphylococcus aureus*. In *E. coli*, Ro 23-9424 produced filaments exclusively and decreased cell growth; cefotaxime produced both filaments and lysis. Like its decomposition product fleroxacin but unlike quinolone esters, Ro 23-9424 also inhibited replicative DNA biosynthesis in *E. coli*. In an *E. coli* strain lacking OmpF, growth continued after addition of Ro 23-9424, decreased after addition of cefotaxime, and stopped immediately after addition of fleroxacin. The results, together with the chemical stability of Ro 23-9424 (half-life, ~3 h at pH 7.4 and 37°C), suggest that in *E. coli* the compound acts initially as a cephalosporin with intrinsic activity comparable to that of cefotaxime but with poorer penetration. Subsequent to the decomposition of Ro 23-9424 to fleroxacin and desacetyl cefotaxime, quinolone activity appears. The in vitro antibacterial activity reflects both mechanisms of action.

Ro 23-9424 is a synthetic, broad-spectrum antibacterial agent consisting of a cephalosporin (cefotaxime) linked at the 3′ position (16) through an ester bond to a fluoroquinolone (fleroxacin) (Fig. 1). Ro 23-9424 combines the antibacterial spectrum and potency of both cefotaxime and fleroxacin (R. N. Jones, A. L. Barry, and C. Thornsberry, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 446, 1988; G. Beskid, V. Fallat, E. Lipschitz, D. McGarry, R. Cleeland, K.-K. Chan, D. Keith, and J. Unowsky, 28th ICAAC, abstr. no. 447, 1988). Most important, its antibacterial activity translates into activity against bacterial infections in animal models (G. Beskid, J. Siebelist, C. McGarry, R. Cleeland, K.-K. Chan, and D. Keith, 28th ICAAC, abstr. no. 448, 1988).

Like other β-lactam antibiotics, cephalosporins exert their antibacterial action by inhibiting specific transpeptidases that are involved in bacterial cell wall biosynthesis (5, 7). These enzymes are conveniently assayed as penicillin-binding proteins (PBPs) because they bind β-lactam antibiotics covalently. In *Escherichia coli*, individual PBPs have specific physiological functions, and their inhibition by β-lactam antibiotics produces distinct morphological effects (19). Quinolones exert their antibacterial action by inhibiting DNA gyrase (6, 21), a unique bacterial enzyme involved in DNA replication (24). This enzyme is assayed either directly, by its ability to supercoil plasmid DNA (12), or indirectly, by its essential function in DNA replication (13).

In the present study, the mechanism whereby Ro 23-9424 exerts its antibacterial effect was investigated and compared with that of its two components as well as with that of structurally related cephalosporins and quinolones. The following properties were specifically examined: (i) binding to PBPs of *E. coli*, *Staphylococcus aureus*, and other bacteria; (ii) effects on replicative DNA biosynthesis in *E. coli*; (iii) effects on cell morphology of *E. coli*; (iv) effects on growth of *E. coli* and *Enterobacter cloacae*, including porin-deficient and β-lactamase-producing strains.

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MATERIALS AND METHODS

Materials. Triton X-100, Trizma base, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), dithiothreitol, ATP, dATP, dCTP, dGTP, dTTP, DNase, and protein molecular weight standards were obtained from Sigma Chemical Co., St. Louis, Mo.; [8-^{14}C]penicillin G (51 μCi/μmol) and [*methyl*-^{3}H]dTTP (50 mCi/μmol) were from Amersham Corp., Arlington Heights, Ill.; reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were from Bio-Rad Laboratories, Richmond, Calif.; Aquasol II and En^{3}Hance were from New England Research Products, Boston, Mass.; XAR-5 X-ray film was from Eastman Kodak Co., Rochester, N.Y.; Whatman 3MM paper, trichloroacetic acid, glycine, bromophenol blue, and all solvents (analytical grade) were from Fisher Scientific Co., Pittsburgh, Pa.; and culture media were from Difco Laboratories, Detroit, Mich.

Organisms. *E. coli* UB1005 and its permeability mutant, DC2 (18), were kindly provided by D. Clark of Southern Illinois University (Carbondale, Ill.); *E. coli* JF568 and its *ompF* mutant, JF703 (14), were gifts from J. Foulds of the National Institutes of Health (Bethesda, Md.); *E. coli* H560 was obtained from B. Bachmann of the *E. coli* Genetic Stock Center, Yale University (New Haven, Conn.); *E. coli* ATCC 25922, *Haemophilus influenzae* ATCC 10211, *Pseudomonas aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *Streptococcus faecalis* ATCC 29212, and *Streptococcus pneumoniae* ATCC 6301 were from the American Type Culture Collection (Rockville, Md.); *E. coli* RC709 (RTEM-1) (11), *E. cloacae* 5699, *Enterobacter cloacae* P99 (23), *Klebsiella pneumoniae* A, *Proteus mirabilis* 2, and *Proteus vulgaris* 6380 were from the Roche culture collection.

Reference antibiotics. Ceftriaxone, desacetyl cefotaxime, fleroxacin, and the methyl esters of norfloxacin, pefloxacin, and oxolinic acid were obtained from Roche Laboratories (Nutley, N.J.). Cefotaxime was from Hoechst-Roussel Pharmaceuticals Inc. (Somerville, N.J.); oxolinic acid was from Sigma; norfloxacin was from Merck & Co., Inc. (Rahway,

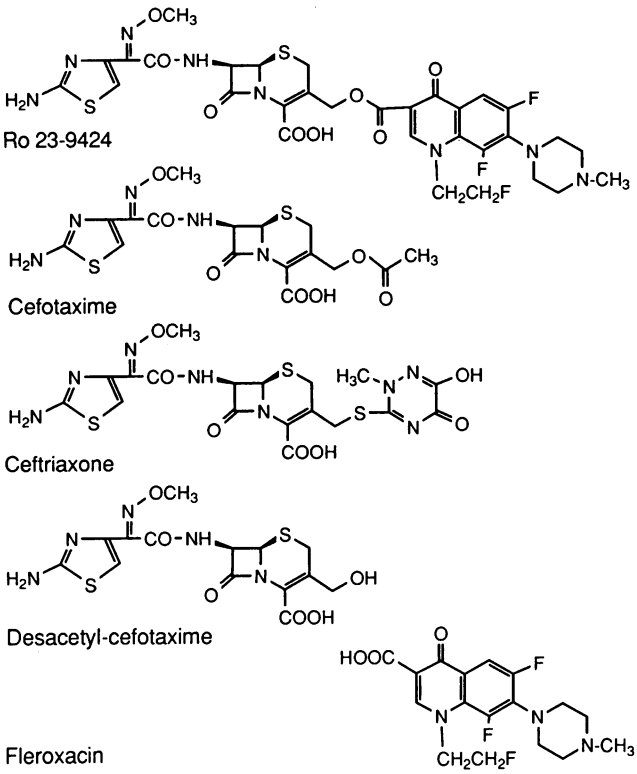

FIG. 1. Structures of Ro 23-9424 and related cephalosporins and quinolones.

N.J.); and pefloxacin was from Rhone-Poulenc Pharmaceuticals (Monmouth Junction, N.J.).

MIC determination. MICs were determined by the broth microdilution method (twofold serial dilution, 100 μl [10^4 CFU] per well, 18 h at 37°C) with Mueller-Hinton broth.

Cell growth and morphology. Cells were incubated (1% inoculum from an overnight culture) with test compound in antibiotic medium 3 at 37°C. Growth was determined turbidimetrically (optical density at 600 nm), while cell morphology was determined (after 3 h of incubation unless indicated otherwise) by light microscopy.

PBP-binding assay. The PBP-binding assay was carried out with Triton X-100-solubilized membranes from sonicated bacteria as previously described (8). Briefly, membranes were incubated for 10 min with the appropriate β-lactam and then for 10 min with 20 μM [^{14}C]penicillin G. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and PBPs were detected by fluorography. PBP binding was measured as inhibition of [^{14}C]penicillin G binding.

TABLE 2. Binding of Ro 23-9424 and related cephalosporins to *S. aureus* ATCC 25923 PBPs

Compound	Concn (μg/ml) required for 90% inhibition of [^{14}C]penicillin G binding to:				MIC (μg/ml)
	PBP 1 (87)[a]	PBP 2 (80)	PBP 3 (75)	PBP 4 (41)	
Ro 23-9424	0.1	0.1	0.1	30	1
Cefotaxime	0.1	0.5	100	100	1
Ceftriaxone	0.5	2	0.5	>100	1
Desacetyl cefotaxime	100	>100	30	>100	4

[a] Number in parentheses is size of PBP in kilodaltons.

Replicative DNA biosynthesis assay. Replicative DNA biosynthesis was measured as the ATP-dependent incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material by toluene-treated *E. coli* H560 cells (13).

RESULTS

The structures of Ro 23-9424, related cephalosporins, and fleroxacin are shown in Fig. 1. The cephalosporins used for comparison were cefotaxime, ceftriaxone, and desacetyl cefotaxime. They all have the same 7β side chain but differ in the 3′ substituent. Desacetyl cefotaxime represented the hydrolysis product of Ro 23-9424. Three of the quinolones used for comparison, fleroxacin, pefloxacin, and norfloxacin, are very similar in structure, fleroxacin being the hydrolysis product of Ro 23-9424.

Binding to PBPs. Tables 1 and 2 show the binding of Ro 23-9424 and related cephalosporins to the PBPs of *E. coli* and *S. aureus*. Ro 23-9424 bound at ≤2 μg/ml to PBPs 1a, 1b, and 3 of *E. coli* and to PBPs 1, 2, and 3 of *S. aureus*, like ceftriaxone and cefotaxime but unlike the Ro 23-9424 decomposition product, desacetyl cefotaxime.

Table 3 shows the binding of Ro 23-9424 to the PBPs of other bacteria. Enterobacteria and *P. aeruginosa* are listed together, as their PBP profiles are very similar (2, 7). When PBPs 1a and 1b were not resolved, a value for PBP 1 is given instead. PBPs were numbered according to the literature: *H. influenzae* by the system of Makover et al. (10), *Streptococcus pneumoniae* by the system of Ellerbrok and Hakenbeck (3), and *Streptococcus faecalis* by the system of Georgopapadakou and Liu (9). Ro 23-9424 bound to PBPs of each organism at concentrations very similar to those of cefotaxime. PBP 3 in gram-negative bacteria and PBPs 1 and 2b in *Streptococcus pneumoniae* were the most sensitive PBPs, binding Ro 23-9424 at ≤0.5 μg/ml. *Streptococcus faecalis* PBPs were insensitive to both Ro 23-9424 and ceftriaxone, with the exception of the nonessential PBP 6, which bound both compounds at ≤0.1 μg/ml.

TABLE 1. Binding of Ro 23-9424 and related cephalosporins to *E. coli* UB1005 PBPs

Compound	Concn (μg/ml) required for 90% inhibition of [^{14}C]penicillin G binding to:						Morphology[a]	MIC[b] (μg/ml)
	PBP 1a (90)[c]	PBP 1b (90)	PBP 2 (66)	PBP 3 (60)	PBP 4 (49)	PBP 5/6 (40)		
Ro 23-9424	0.1	2	100	0.1	10	>100	F	0.2 (0.02)
Cefotaxime	0.1	0.5	100	0.1	100	>100	F, L	0.03 (0.02)
Ceftriaxone	0.5	2	10	0.1	100	>100	F, L	0.06 (0.02)
Desacetyl cefotaxime	≥100	≥100	>100	10	100	>100	F	0.5 (0.2)

[a] F, Filaments; L, lysis.
[b] Numbers in parentheses refer to MICs for DC2, a permeability mutant of strain UB1005 (18).
[c] Number in parentheses is size of PBP in kilodaltons.

TABLE 4. Effects of Ro 23-9424 and related quinolones on replicative DNA biosynthesis in *E. coli* H560

Compound	IC_{50}[a] (μg/ml)	MIC (μg/ml) for *E. coli*:	
		UB1005[b]	25922
Ro 23-9424	8	0.1	0.1
Fleroxacin	1	1	0.03
Pefloxacin	2	2	0.06
Pefloxacin methyl ester	>100	ND[c]	ND
Norfloxacin	0.5	0.2	0.03
Norfloxacin methyl ester	>100	128	16
Oxolinic acid	7	8	0.2
Oxolinic acid methyl ester	>100	ND	ND

[a] 50% inhibitory concentration.
[b] A nalidixic acid-resistant strain (1).
[c] ND, Not determined.

Cell morphology. The effects of Ro 23-9424 and related cephalosporins on the cell morphology of *E. coli* are indicated in Table 1. Ro 23-9424 and desacetyl cefotaxime produced exclusively filaments, while cefotaxime and ceftriaxone produced both filaments and lysis.

Replicative DNA biosynthesis. The effects of Ro 23-9424, related quinolones, and their esters on replicative (ATP-dependent) DNA biosynthesis were used as a convenient indicator of the effects on DNA gyrase activity (17, 20). Ro 23-9424 inhibited replicative DNA biosynthesis in *E. coli*, with an apparent 50% inhibitory concentration eightfold higher than that of its decomposition product, fleroxacin, while all other quinolone esters were inactive (Table 4).

In a separate experiment, the 50% inhibitory concentration of Ro 23-9424 was determined as a function of time under assay conditions (30°C, pH 8.0). In that experiment, when the assay time was reduced to 10 min to minimize decomposition during assay, the 50% inhibitory concentration decreased from ~100 to ~10 μg/ml after 40 min of incubation: The latter value corresponds to 1 μg of free fleroxacin per ml (i.e., 10% decomposition), which extrapolates to a half-time of decomposition of ~3 h.

Membrane permeation. To determine the extent and the pathway of entry of intact Ro 23-9424 in *E. coli*, its effect on cell growth was examined for periods of up to 100 min (Fig. 2). Ro 23-9424 inhibited the growth of wild-type *E. coli* less than cefotaxime or fleroxacin (Fig. 2A), suggesting decreased penetration. Its effects on the growth of a porin-deficient strain, JF703, were much reduced (Fig. 2B), suggesting that penetration occurs through porins (15).

Effect of β-lactamases. The effects of two major gram-negative β-lactamases, the R_{TEM} and P99 enzymes (22), on fleroxacin release from Ro 23-9424 were determined by comparing the effects of Ro 23-9424 and fleroxacin on growth of *E. coli* and *E. cloacae* carrying the two enzymes (Fig. 2 and 3). It was expected that β-lactamase hydrolysis of Ro 23-9424 would result in the expulsion of the 3′ substituent (4) and the appearance of free fleroxacin and would thus be manifested as increased growth inhibition. However, neither R_{TEM} (Fig. 2C) nor P99 (Fig. 3B) potentiated the growth-inhibitory effects of Ro 23-9424, although in both cases the growth-inhibitory effects of cefotaxime were reduced, suggesting sensitivity to β-lactamase.

DISCUSSION

Ro 23-9424 is a cephalosporin 3′-quinolone ester having broad-spectrum antibacterial activity. In the present study it was found to act both as a cephalosporin and as a quinolone

TABLE 3. Binding of Ro 23-9424 and cefotaxime to PBPs of different bacteria

Organism	Compound	Concn required for 90% inhibition of [^{14}C]penicillin binding to:												
		PBP 1[a]	PBP 1a	PBP 1b	PBP 2a (81 kDa)	PBP 2b (77 kDa)	PBP 2	PBP 3[b]	PBP 4[c]	PBP 5/6[d]	PBP 5/6	PBP 6[e]	PBP 7	PBP 8
Enterobacter cloacae 29528	Ro 23-9424		>100	>100			≥100	0.5	100	≥100				
	Cefotaxime		>100	>100			100	0.5	100	≥100				
Klebsiella pneumoniae A	Ro 23-9424		0.5	0.5			30	0.1	10	≥100				
	Cefotaxime		2.0	2.0			30	0.1	10	≥100				
Proteus mirabilis 2	Ro 23-9424		0.5	100			2	0.1	2	>100				
	Cefotaxime		0.5	100			10	0.1	10	>100				
Proteus vulgaris 6380	Ro 23-9424		>100	10			30	0.1	2	>100				
	Cefotaxime			10			30	0.1	2	>100				
Pseudomonas aeruginosa 27853	Ro 23-9424		0.1	0.5			ND[f]	0.1	>100	>100				
	Cefotaxime		0.1	2.0			ND	0.1	30	>100				
Haemophilus influenzae 10211	Ro 23-9424	10					30	10	0.1		10	>100	>100	0.1
	Cefotaxime	10					30	10	0.1		10	>100	>100	0.1
Streptococcus pneumoniae 6301	Ro 23-9424	0.5			100	0.1	0.1							
	Cefotaxime	0.5			>100	0.1	0.1							
Streptococcus faecalis 29212	Ro 23-9424	>100					ND	>100	30		0.1			
	Cefotaxime	>100					>100	>100	30		0.1			

[a] 90 kDa in *H. influenzae* and *Streptococcus pneumoniae*.
[b] 75 kDa in *H. influenzae*; 43 kDa in *Streptococcus pneumoniae*; 78 kDa in *Streptococcus faecalis*.
[c] 68 kDa in *H. influenzae* and 74 kDa in *Streptococcus faecalis*.
[d] 64 kDa in *H. influenzae* and 42 kDa in *Streptococcus faecalis*.
[e] 48 kDa in *H. influenzae* and 35 kDa in *Streptococcus faecalis*.
[f] ND, Not determined.

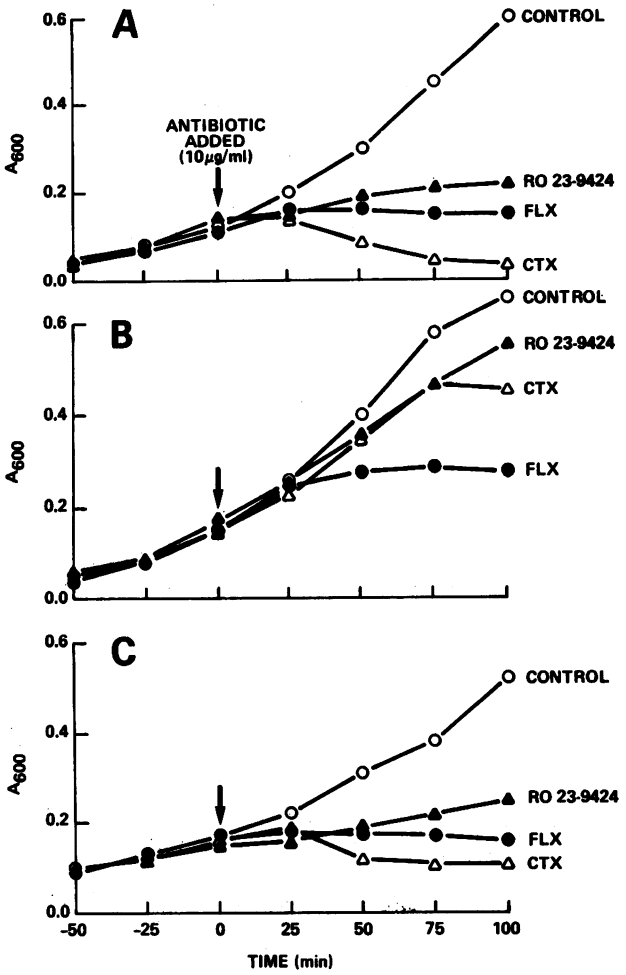

FIG. 2. Effect of Ro 23-9424, cefotaxime (CTX), and fleroxacin (FLX) on the growth of wild-type JF568 (A), porin-deficient JF703 (B), and β-lactamase-producing RC709 (C) *E. coli*.

prodrug. As a cephalosporin, it bound to essential PBPs of both gram-positive and gram-negative bacteria, including *P. aeruginosa*. Its modest activity against this organism is probably due to permeability limitations. On the other hand, its modest activity toward *Streptococcus faecalis* is probably due to poor intrinsic activity. Although it bound to both PBPs 1 and 3 of *E. coli*, it produced exclusively filaments in that organism. This suggests a low steady-state concentration of Ro 23-9424 in the periplasmic state, due to poor outer-membrane permeability, allowing binding only to PBP 3.

As a quinolone prodrug, Ro 23-9424 releases fleroxacin, which then inhibits DNA gyrase. The intact Ro 23-9424 is probably inactive as a quinolone. The in vitro release of fleroxacin from Ro 23-9424 is largely nonenzymatic; two common β-lactamases, R_{TEM} and P99, did not increase fleroxacin release. The in vivo release and bioavailability of fleroxacin are more relevant clinically, but far more complex; they are functions of the pharmacokinetics of both Ro 23-9424 and fleroxacin and are influenced by such host factors as liver esterases.

In conclusion, Ro 23-9424 acts in *E. coli* initially as a cephalosporin, with intrinsic activity comparable to that of cefotaxime but with poorer penetration. Quinolone activity appears subsequent to the decomposition of Ro 23-9424 to fleroxacin and desacetyl cefotaxime. The in vitro antibacterial activity reflects both mechanisms of action.

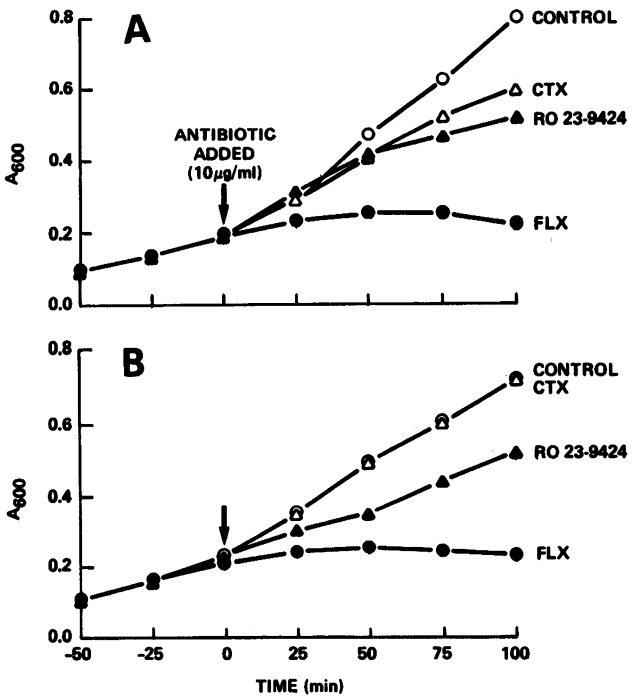

FIG. 3. Effect of Ro 23-9424, cefotaxime (CTX), and fleroxacin (FLX) on the growth of wild-type 5699 (A) and β-lactamase-producing P99 (B) *E. cloacae*.

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