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β-Lactamase Hydrolysis of Cephalosporin 3′-Quinolone Esters, Carbamates, and Tertiary Amines

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The β-lactam hydrolysis of five cephalosporin 3′-quinolones (dual-action cephalosporins) by three gram-negative β-lactamases was examined. The dual-action cephalosporins tested were the ester Ro 23-9424; the carbamates Ro 25-2016, Ro 25-4095, and Ro 25-4835; and the tertiary amine Ro 25-0534. Also tested were cephalosporins with similar side chains (cefotaxime, desacetylcefotaxime, cephalothin, cephacetrile, and Ro 09-1227 [SR 0124]) and standard β-lactams (penicillin G, cephaloridine). The β-lactamases used were the plasmid-mediated TEM-1 and TEM-3 enzymes and the chromosomal AmpC. The cephacetrile-related compounds Ro 25-4095 and Ro 25-4835 were hydrolyzed by all three β-lactamases with catalytic efficiencies (relative to penicillin G) ranging from ~5 (TEM-1, AmpC) to ~25 (TEM-3). The cephalothin-related Ro 25-2016 was also hydrolyzed by all three β-lactamases, particularly the AmpC enzyme (relative catalytic efficiency, 110). The cefotaxime-related compounds Ro 25-0534 and Ro 23-9424 were hydrolyzed to any significant extent only by the TEM-3 enzyme (relative catalytic efficiencies, 1.2 and 4.7, respectively).

In recent years, a number of compounds which consist of a cephalosporin covalently linked at the 3′ position to a quinolone via an ester, carbamate, or tertiary amine linkage have been synthesized (1–3, 5). These compounds have a dual mechanism of action (hence the name dual-action cephalosporins [DACs]), reflecting the actions of both the β-lactam and the quinolone components; they bind to penicillin-binding proteins and inhibit DNA gyrase (1–3, 10, 18). In growing *Escherichia coli* devoid of β-lactamase, the esters act as cephalosporins when intact, while the carbamates and tertiary amines act primarily as quinolones (8, 9).

Theoretically, cephalosporin 3′-quinolones may release free quinolone directly, by hydrolysis of the cephalosporin-quinolone linkage, or indirectly, subsequent to opening of the β-lactam ring (6, 14). The former reaction is chemical, while the latter reaction is enzymatic and is catalyzed primarily by β-lactamases. In the study described in this report, we examined the hydrolysis of cephalosporin 3′-quinolones and reference compounds by three gram-negative β-lactamases. The first, TEM-1, is plasmid mediated and is the most common β-lactamase in gram-negative bacteria. The second, TEM-3, is a cefotaxime-hydrolyzing enzyme derived from TEM-2 (itself a variant of TEM-1). The third, AmpC, is a chromosomally encoded β-lactamase similar to those responsible for β-lactam resistance in *Enterobacter cloacae* and *Pseudomonas aeruginosa*. β-Lactam hydrolysis of the cephalosporin 3′-quinolones by any of the three enzymes would be expected to release free quinolone (14).

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA), egg white lysozyme, EDTA, and Trizma base were obtained from Sigma Chemical Co. (St. Louis, Mo.); DEAE cellulose (DE-52) and carboxymethyl cellulose (CM-52) were from Pharmacia LKB Biotechnology (Piscataway, N.J.); potassium and potassium iodide were from Fisher Scientific Co. (Pittsburgh, Pa.).

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Antibiotics. Desacetylcefotaxime (Ro 24-2414), Ro 09-1227 (SR 0124), cephacetrile, and all DACs were from Antiinfective Chemistry, Roche Laboratories. Cefotaxime was from Hoechst-Roussel Pharmaceuticals Inc. (Somerville, N.J.), cephalothin and penicillin G were from Lilly Research Laboratories (Indianapolis, Ind.), cephaloridine was from Glaxo Group Research, Ltd. (Greenford, United Kingdom), and ampicillin was from Wyeth Laboratories (Philadelphia, Pa.).

Bacterial strains and growth conditions. *E. coli* MK47, a K-12 strain containing the plasmid-encoded TEM-1 β-lactamase (13), was a gift from M. Gellert of the National Institutes of Health (Bethesda, Md.); *E. coli* CF204, a K-12 C600 strain containing the plasmid-encoded CTX-1 (TEM-3) β-lactamase (21), was a gift from D. Sirot of Clermont Ferrand Hospital (France); and *E. coli* TE18, a K-12 strain containing the *ampC* gene and an overproducer of chromosomal β-lactamase (17), was a gift from S. Normark of Washington University (St. Louis, Mo.).

Strains were grown in Luria broth at 37°C to the late logarithmic phase, with the exception of *E. coli* MK47, which was grown at 30°C in Luria broth containing 0.5% glucose, 5 mM potassium phosphate, 1 μg of biotin per ml, and 10 μg of ampicillin per ml as described previously (13). Ampicillin (50 μg/ml) was also included in the growth medium of *E. coli* CF204 to maintain the plasmid.

β-Lactamase purification. In the first step, β-lactamases were released from the periplasm by osmotic shock (15), a procedure essentially the same for all three enzymes. Briefly, *E. coli* cells (typically, 15 g for TEM-1, 1 to 2 g for TEM-3, 15 g for the AmpC enzyme) were harvested, washed with 20 mM Tris-HCl (pH 7.5), resuspended in 5 to 10 volumes of 20 mM Tris-HCl (pH 7.5) containing 20% sucrose and 1 mM EDTA, and incubated at room temperature for 10 min. With cell pastes larger than 15 g, cells were resuspended in 2 volumes of the buffer described above, and the incubation time was increased to 30 min. After centrifugation at 13,000 × *g* for 10 min at 4°C, the pellet was immediately resuspended in 2 volumes of cold deionized water and the mixture was stirred vigorously for 10 min at 4°C. After centrifugation at 13,000 × *g* for 10 min, the supernatant was retained and the activity was measured. To the supernatant, 1 M Tris-HCl (at pH 7.5 for

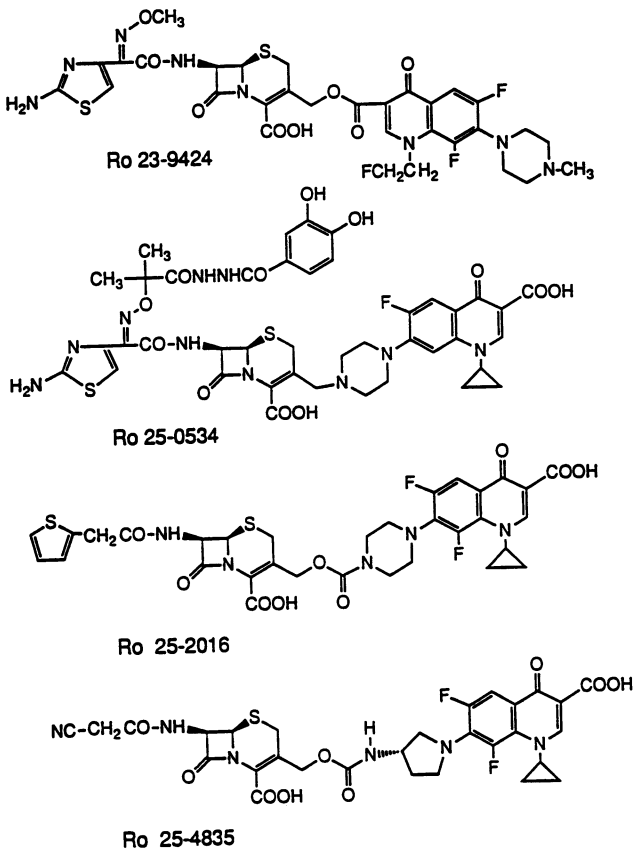

FIG. 1. Structures of DACs used in the study. Ro 25-4095 is the racemate of Ro 25-4835.

TEM-1 and TEM-3 or at pH 7.0 for AmpC) was added to a final concentration of 10 mM.

TEM-1 (pI, 5.4 [4]) and TEM-3 (pI, 6.3 [4]) β-lactamases were further purified, as follows (7, 22). The supernatant from the osmotic shock step was loaded onto a DE-52 column (1- to 2-ml column volume per 1 g of original cell paste) equilibrated with 10 mM Tris-HCl (pH 7.5). The column was washed with 3 volumes of equilibration buffer and was eluted with 100 mM Tris-HCl (pH 7.5). The eluate was concentrated 5-fold (TEM-3) or 20-fold (TEM-1) by centrifugation through a Centricon-10 (Amicon) filter (protein concentration, ~1 or ~10 mg/ml, respectively). If not used immediately, it was stored at 4°C. In that form, the enzyme was stable for at least 10 days.

AmpC (pI, 9.2 [4]) β-lactamase was further purified as follows (11, 12). The supernatant from the osmotic shock step was loaded onto a CM-52 column equilibrated with 10 mM Tris-HCl (pH 7.0). The column was washed with 3 volumes of equilibration buffer and was eluted with 100 mM Tris-HCl (pH 7.0). The eluate was concentrated 10-fold by centrifugation through a Centricon-10 filter (protein concentration, ~0.1 mg/ml), BSA was added to a final concentration of 1 mg/ml, and the solution was stored at 4°C. In that form, the enzyme was stable for at least 12 days.

β-Lactamase assay. β-Lactamase activity was assayed in most cases spectrophotometrically with a Beckman DU 70 instrument. The assay was carried out at 30°C in 10 mM sodium phosphate (pH 7.0). β-Lactam hydrolysis was moni-

TABLE 1. Differential absorption maxima and extinction coefficients of DACs and related compounds and their hydrolysis products

Compound	λ (nm)	Δε (M^{-1} cm^{-1})
Penicillin G	233	1,100
Cephaloridine	260	8,000
Ro 23-9424	295	11,000
Cefotaxime	264	7,500
Desacetylcefotaxime	270	5,000
Ro 25-0534	272	6,955
Ro 09-1227 (SR 0124)	295	5,825
Ro 25-2016	275	2,800
Cephalothin	260	7,000
Ro 25-4095	291	10,000
Ro 25-4835	291	10,000
Cephacetrile	262	6,800

TABLE 2. Kinetic parameters of DACs and related compounds for TEM-1 β-lactamase

Compound	V_{max} (mmol/min/mg)	Relative V_{max}	K_m (μM)	Relative V_{max}/K_m
Penicillin G	124	100	24	100
Cephaloridine	168	135	690	4.7
Ro 23-9424	0.03	0.024	113	0.005
Cefotaxime	ND[a]	ND	10,000	ND
Desacetylcefotaxime	ND	ND	>2,000	ND
Ro 25-0534	0.002	0.0016	48	0.0008
Ro 09-1227 (SR 0124)	ND	ND	>2,000	ND
Ro 25-2016	52	42	26	39
Cephalothin	9.7	7.8	130	1.4
Ro 25-4095	5.4	4.4	15	7.0
Ro 25-4835	7.0	5.6	38	3.6
Cephacetrile	21.8	17	1,460	0.29

[a] ND, not determined (no hydrolysis detected).

TABLE 3. Kinetic parameters of DACs and related compounds for TEM-3 β-lactamase

Compound	V_{max} (mmol/min/mg)	Relative V_{max}	K_m (μM)	Relative V_{max}/K_m
Penicillin G	3.6	100	1.1	100
Cephaloridine	7.3	202	23	9.7
Ro 23-9424	1.4	42	9.9	4.7
Cefotaxime	18	500	39	14.1
Desacetylcefotaxime	7.2	200	48	4.6
Ro 25-0534	0.5	14	13	1.2
Ro 09-1227 (SR 0124)	0.43	12	68	0.19
Ro 25-2016	2.3	64	1.0	70.4
Cephalothin	2.5	69	3.3	23.0
Ro 25-4095	3.5	97	3.7	28.8
Ro 25-4835	2.4	67	4.0	18.4
Cephacetrile	4.8	131	36	4.1

TABLE 4. Kinetic parameters of DACs and related compounds for AmpC β-lactamase

Compound	V_{max} (mmol/min/mg)	Relative V_{max}	K_m (μM)	Relative V_{max}/K_m
Penicillin G	68	100	3.0	100
Cephaloridine	194	285	236	3.6
Ro 23-9424	0.096	0.14	38	0.011
Cefotaxime	0.035	0.51	0.3	5.1
Desacetylcefotaxime	0.8	1.2	1.3	2.7
Ro 25-0534	0.37	0.54	88	0.02
Ro 09-1227 (SR 0124)	<0.01[a]	ND[b]	ND	ND
Ro 25-2016	276	406	11	110
Cephalothin	350	515	36	43
Ro 25-4095	23	34	11	9.2
Ro 25-4835	13	19	11	5.2
Cephacetrile	67	98	97	3.0

[a] Ro 09-1227 concentration, 0.1 mM.
[b] ND, not determined (no hydrolysis detected).

tored at wavelengths selected from the maximum absorbance difference between hydrolyzed and unhydrolyzed β-lactams (19). The kinetic parameters K_m and V_{max} were determined from Lineweaver-Burk plots of the initial velocities of hydrolysis at various substrate concentrations between 0.33 and 4 times the K_m. In cases of low K_m values (i.e., ≤2 μM), the values were obtained directly from the time course of the hydrolysis data (16). In cases of high K_m values (initial absorbance reading, ≥2), the assay was carried out according to the iodometric method of Sargent (20).

RESULTS AND DISCUSSION

The structures of the DACs tested for β-lactamase hydrolysis are shown in Fig. 1. Also tested were cephalosporins with similar side chains (cefotaxime, desacetylcefotaxime, cephalothin, cephacetrile, and Ro 09-1227) and standard compounds (penicillin G and cephaloridine). The differential absorption maxima and extinction coefficients of all compounds used in the study and their hydrolysis products are given in Table 1, while the kinetic parameters obtained with the three β-lactamases are given in Tables 2, 3 and 4.

TEM-1 β-lactamase hydrolyzed only the cephalothin-related Ro 25-2016 and the cephacetrile-related Ro 25-4095 and Ro 25-4835 to any significant extent (Table 1). Their catalytic efficiencies (V_{max}/K_m) relative to that of penicillin G were 39, 7.0, and 3.6, respectively, reflecting lower hydrolysis rates rather than lower affinities. Interestingly, the affinities for all five DACs were similar to that of penicillin G (K_ms, $\sim 10^{-5}$ M).

TEM-3 β-lactamase hydrolyzed all five DACs with relative catalytic efficiencies ranging from 1.2 for the SR 0124-related Ro 25-0534 to ~70 for the cephalothin-related Ro 25-2016 (Table 2). Differences in catalytic efficiencies reflected different affinities or different rates of hydrolysis, or both.

AmpC β-lactamase hydrolyzed primarily the cephalothin-related Ro 25-2016, with a catalytic efficiency similar to that of penicillin G (Table 3). It also hydrolyzed the cephacetrile-related Ro 25-4095 and Ro 25-4835, but with significantly lower catalytic efficiencies, reflecting lower hydrolysis rates. The SR 0124-related Ro 25-0534 and the cefotaxime-related Ro 23-9424 were marginally hydrolyzed, reflecting both lower affinities and lower hydrolysis rates.

In conclusion, of the DACs examined, the ester Ro 23-9424 and the tertiary amine Ro 25-0534 were the most stable to β-lactamase hydrolysis. Recall that the former compound undergoes chemical hydrolysis of the ester linkage with a half-life of ~3 h at 37°C and pH 7.4, while the latter compound undergoes hydrolysis of the tertiary amine linkage with a half-life of 120 h under the same conditions. The three carbamates (Ro 25-2016, Ro 25-4095, and Ro 25-4835), on the other hand, were susceptible to hydrolysis by all three β-lactamases. In view of their chemical stability (half-life, ~20 h), they are most likely to release quinolone in gram-negative bacteria through β-lactamase-mediated hydrolysis of the β-lactam ring, as has been proposed previously (1, 14).

REFERENCES

1. **Albrecht, H. A., G. Beskid, K.-K. Chan, J. G. Christenson, R. Cleeland, K. H. Deitscher, N. H. Georgopapadakou, D. D. Keith, D. L. Pruess, J. Sepinwall, A. C. Specian, Jr., R. L. Then, M. Weigele, K. F. West, and R. Yang.** 1990. Cephalosporin 3′-quinolone esters with a dual mode of action. J. Med. Chem. **33**:77–86.
2. **Albrecht, H. A., G. Beskid, J. G. Christenson, J. W. Durkin, V. Fallat, N. H. Georgopapadakou, D. D. Keith, F. M. Konzelmann, E. R. Lipschitz, D. H. McGarry, J. Siebelist, C. C. Wei, M. Weigele, and R. Yang.** 1991. Dual-action cephalosporins: cephalosporin 3′-quaternary ammonium quinolones. J. Med. Chem. **34**:669–675.
3. **Albrecht, H. A., G. Beskid, J. G. Christenson, N. H. Georgopapadakou, D. D. Keith, F. M. Konzelmann, D. L. Pruess, P. L. Rossman, and C.-C. Wei.** 1991. Dual-action cephalosporins: cephalosporin 3′-quinolone carbamates. J. Med. Chem. **34**:2857–2864.
4. **Bush, K.** 1989. Classification of β-lactamases: groups 1, 2a, 2b, and 2b′. Antimicrob. Agents Chemother. **33**:264–270.
5. **Demuth, T. P., Jr., R. E. White, R. A. Titjen, R. J. Storrin, J. R. Skuster, J. A. Andersen, C. C. McOsker, R. Freedman, and F. J. Rourke.** 1991. Synthesis and antibacterial activity of new C-10 quinolonyl-cephem esters. J. Antibiot. **44**:200–209.
6. **Faraci, W. S., and R. F. Pratt.** 1986. Mechanism of inhibition of RTEM-2 β-lactamase by cephamycins: relative importance of the 7a-methoxy group and the 3′ leaving group. Biochemistry **25**:2934–2941.
7. **Fisher, J., J. G. Belasco, S. Khosla, and J. R. Knowles.** 1980. β-Lactamase proceeds via an acyl-enzyme intermediate. Interaction of the *Escherichia coli* RTEM enzyme with cefoxitin. Biochemistry **19**:2895–2900.
8. **Georgopapadakou, N. H., and A. Bertasso.** 1991. Effects of quinolones on nucleoid segregation in *Escherichia coli*. Antimicrob. Agents Chemother. **35**:2645–2648.
9. **Georgopapadakou, N. H., and A. Bertasso.** 1993. Mechanism of action of cephalosporin 3′-quinolone esters, carbamates, and tertiary amines in *Escherichia coli*. Antimicrob. Agents Chemother. **37**:559–565.
10. **Georgopapadakou, N. H., A. Bertasso, K.-K. Chan, J. S. Chapman, R. Cleeland, L. M. Cummings, B. A. Dix, and D. D. Keith.** Mode of action of the dual-action cephalosporin Ro 23-9424. Antimicrob. Agents Chemother. **33**:1067–1071.
11. **Hennessey, T. D., and M. H. Richmond.** 1968. The purification and some properties of a β-lactamase (cephalosporinase) synthesized by *Enterobacter cloacae*. Biochem. J. **109**:469–473.
12. **Hirai, K., S. Iyobe, M. Inoue, and S. Mitsuhashi.** 1980. Purification and properties of a new β-lactamase from *Pseudomonas cepacia*. Antimicrob. Agents Chemother. **17**:355–358.
13. **Mizuuchi, K., M. Mizuuchi, M. H. O'Dea, and M. Gellert.** 1984. Cloning and simplified purification of *Escherichia coli* DNA gyrase A and B proteins. J. Biol. Chem. **259**:9199–9201.
14. **Mobashery, S., S. A. Lerner, and M. Johnston.** 1986. Conscripting β-lactamase for use in drug delivery. Synthesis and biological activities of a cephalosporin C_{10}-ester of an antibiotic dipeptide. J. Am. Chem. Soc. **108**:1685–1686.
15. **Neu, H. C., and L. A. Heppel.** 1965. The release of enzymes from

Escherichia coli by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240:**3685–3692.

16. **Nikaido, H., W. Liu, and A. Y. Rosenberg.** 1990. Outer membrane permeability and β-lactamase stability of dipolar ionic cephalosporins containing methoxyimino substituents. Antimicrob. Agents Chemother. **34:**337–342.
17. **Nikaido, H., and S. Normark.** 1987. Sensitivity of *Escherichia coli* to various β-lactams is determined by the interplay of outer membrane permeability and degradation by periplasmic β-lactamases: a quantitative predictive treatment. Mol. Microbiol. **1:**29–36.
18. **Pace, J., A. Bertasso, and N. H. Georgopapadakou.** 1991. *Escherichia coli* resistant to cephalosporins and quinolones is still susceptible to the cephalosporin-quinolone ester Ro 23-9424. Antimicrob. Agents Chemother. **35:**910–915.
19. **Samuni, A.** 1975. A direct spectrophotometric assay and determination of Michaelis constants for the β-lactamase reaction. Anal. Biochem. **63:**17–26.
20. **Sargent, M. S.** 1968. Rapid fixed-time assay for penicillinase. J. Bacteriol. **95:**1493–1494.
21. **Sirot, D., J. Sirot, R. Labia, A. Morand, P. Courvalin, A. Darfeuille-Michaud, R. Perroux, and R. Cluzel.** 1987. Transferable resistance to third generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel β-lactamase. J. Antimicrob. Chemother. **20:**323–334.
22. **Zafaralla, G., E. K. Manavathu, S. A. Lerner, and S. Mobashery.** 1992. Elucidation of the role of arginine-244 in the turnover processes of class A β-lactamases. Biochemistry **31:**3847–3852.