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In Vitro Activity of AT-4140 against Quinolone- and Methicillin-Resistant *Staphylococcus aureus*

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Eighty-nine clinical isolates of *Staphylococcus aureus* that were resistant to both ciprofloxacin (MIC, ≧3.13 μg/ml) and methicillin (MIC, ≧12.5 μg/ml) were divided into two groups with respect to their susceptibilities to AT-4140. Most isolates that were moderately resistant to ciprofloxacin (MICs, 3.13 to 12.5 μg/ml) or ofloxacin (MICs, 0.78 to 6.25 μg/ml) were susceptible to AT-4140 (MICs, 0.05 to 0.2 μg/ml). Most isolates that were highly resistant to ciprofloxacin (MIC, ≧25 μg/ml) or ofloxacin (MIC, ≧12.5 μg/ml) were resistant to AT-4140 (MICs, 3.13 to 25 μg/ml). The appearance of spontaneous single-step, quinolone-resistant mutants of *S. aureus* P-20, a methicillin-resistant isolate, was more frequent than was that of *S. aureus* 209P JC-1, a susceptible laboratory strain. Spontaneous single-step, quinolone-resistant mutants of P-20 were not selected by AT-4140, and those selected by existing fluoroquinolones were susceptible to AT-4140. Spontaneous double-step, quinolone-resistant mutants of P-20 were selected by various fluoroquinolones. All second-step mutants selected by AT-4140 or ofloxacin from P-20-C, a spontaneous single-step mutant of P-20 selected by ciprofloxacin, were resistant to all the quinolones. All second-step mutants selected by norfloxacin were resistant to all existing fluoroquinolones but were less resistant to AT-4140. There was a close resemblance between the resistance profiles of spontaneous quinolone-resistant mutants and those of clinically isolated quinolone- and methicillin-resistant *S. aureus*.

The widespread incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious problem in antibacterial chemotherapy (9). MRSA is intrinsically resistant to β-lactam antibiotics because of the production of penicillin-binding protein 2′, a new penicillin-binding protein, with a low affinity for β-lactams. β-Lactam treatment of MRSA infections appears to be less effective (1, 18, 19). In addition, clinical isolates of MRSA tend to have multiple antibiotic resistances (7, 9).

Ciprofloxacin possesses a wide spectrum of activity against both gram-positive and gram-negative bacteria (14, 20) and potent activity against MRSA (16). Ciprofloxacin therapy for MRSA infections has been attempted (17); however, ciprofloxacin seems to select quinolone-resistant mutants of MRSA (10), and the emergence of ciprofloxacin-resistant MRSA has diminished the efficacy of ciprofloxacin therapy (6, 13).

AT-4140 is a new fluoroquinolone antibacterial agent for oral use. One of its distinctive properties is improved activity against gram-positive cocci (8, 11). Therefore, the activity of AT-4140 against ciprofloxacin-resistant MRSA and the frequency of *S. aureus* mutants that are resistant to AT-4140 were examined in comparison with those of the existing fluoroquinolones, ciprofloxacin, ofloxacin, and norfloxacin.

MATERIALS AND METHODS

Drugs. AT-4140 was obtained from Dainippon Pharmaceutical Co., Ltd., Osaka, Japan; ciprofloxacin was from Bayer Yakuhin, Ltd., Osaka, Japan; ofloxacin and nalidixic acid were from Daiichi Seiyaku Co., Ltd., Tokyo, Japan; norfloxacin was from Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan; novobiocin was from Sigma Chemical Co., St. Louis, Mo.; minocycline was from Lederle (Japan), Ltd., Tokyo, Japan; vancomycin was from Shionogi & Co., Ltd., Osaka, Japan; and methicillin was from Banyu Pharmaceutical Co., Ltd., Tokyo, Japan. Working solutions of each antibacterial agent were prepared before use.

Bacterial strains. Eighty-nine isolates of *S. aureus* resistant to both ciprofloxacin (MIC, ≧3.13 μg/ml) and methicillin (MIC, ≧12.5 μg/ml) were collected from various hospitals in Japan. *S. aureus* 209P JC-1 was a laboratory strain that was susceptible to various antibacterial agents. *S. aureus* MS16008, MS16373, MS16415, and MS16427 were randomly selected from clinical isolates that were susceptible to both ciprofloxacin and methicillin. *S. aureus* MS16023, MS16086, P-5, P-20, and P-86 were randomly selected from clinical isolates of MRSA that were susceptible to quinolones. *S. aureus* P-20-C was obtained in this study as a spontaneous, single-step quinolone-resistant mutant of P-20 selected by ciprofloxacin at a concentration of 1.56 μg/ml.

Drug susceptibility testing. Drug susceptibility was tested by the twofold serial agar dilution method with Sensitivity Disk Agar-N (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The test organisms were cultured overnight in Sensitivity Test Broth (Nissui) without shaking and were diluted with phosphate-buffered saline containing 0.01% gelatin. By using an inoculation apparatus (Microplanter; Sakuma Seisakusho, Tokyo, Japan), a 5-μl portion of the dilution was spotted onto agar plates containing antibacterial agents at graded concentrations. The final inoculum was approximately 5×10^3 CFU per spot. The MIC was determined after an overnight incubation at 37°C.

Mutant frequency test. Test organisms were grown in Sensitivity Test Broth at 37°C with shaking, and 0.1 ml of the overnight cultures or a 10-fold dilution of the overnight culture with saline was spread onto Sensitivity Disk Agar-N plates containing each fluoroquinolone at concentrations of 4, 8, and 16 times the corresponding MIC. The frequency of

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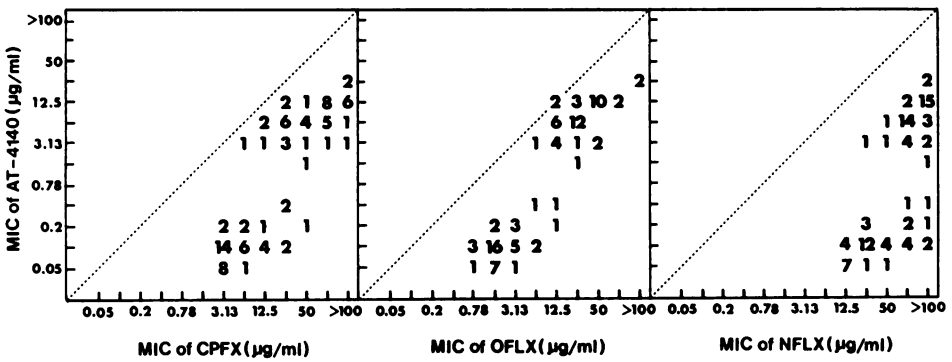

FIG. 1. Relationship between susceptibilities of ciprofloxacin- and methicillin-resistant *S. aureus* to AT-4140 and those to ciprofloxacin (CPFX), ofloxacin (OFLX), and norfloxacin (NFLX). The numbers in the figures are the number of isolates with the corresponding MICs. Isolates of group 1 were resistant to ciprofloxacin, ofloxacin, and norfloxacin but were susceptible to AT-4140 at concentrations below 0.78 μg/ml. Isolates of group 2 were resistant to all the fluoroquinolones, including AT-4140 (MIC of AT-4140, >0.78 μg/ml).

spontaneous mutations selected by each fluoroquinolone was calculated as the ratio of the number of cells growing on drug agar to the number of inoculated cells after incubation at 37°C for 40 h. The mutants which appeared on the agar plate containing each fluoroquinolone at the highest concentration that allowed the growth of spontaneous mutants were transferred to Sensitivity Test Broth. Drug susceptibilities of the spontaneous mutants were tested as described above.

RESULTS

Drug susceptibilities of clinical isolates. MICs of methicillin were ≧100, 50, 25, and 12.5 μg/ml for 75, 4, 6, and 4, of the 89 isolates of ciprofloxacin-resistant MRSA tested in this study, respectively. The relationship between the susceptibilities of the isolates to AT-4140 and to ciprofloxacin, ofloxacin, and norfloxacin are shown in Fig. 1. Among the fluoroquinolones tested in this study, AT-4140 was the most active agent against almost all isolates. The isolates were divided into two groups with respect to their susceptibilities to AT-4140. Of the 89 isolates, 43 (48%) were susceptible to AT-4140 (MICs, 0.05 to 0.39 μg/ml), and the others were resistant to AT-4140 (MICs, 1.56 to 25 μg/ml). Although most isolates that were highly resistant to ciprofloxacin (MIC, ≧25 μg/ml) or ofloxacin (MIC, ≧12.5 μg/ml) were also resistant to AT-4140 (MICs, 3.13 to 25 μg/ml), most isolates that were moderately resistant to ciprofloxacin (MICs, 3.13 to 12.5 μg/ml) or ofloxacin (MICs, 0.78 to 6.25 μg/ml) were susceptible to AT-4140 (MICs, 0.05 to 0.2 μg/ml). While 54 of 89 isolates (61%) were highly resistant to norfloxacin (MIC, ≧100 μg/ml), 11 of the 54 isolates (20%) were susceptible to AT-4140 (MICs, 0.05 to 0.39 μg/ml). Thus, there was a difference in the susceptibility profile between AT-4140 and those of the existing fluoroquinolones among the clinical isolates of ciprofloxacin-resistant MRSA tested.

Frequency of spontaneous quinolone-resistant mutation. The ability of AT-4140, ciprofloxacin, ofloxacin, and norfloxacin to select quinolone-resistant mutants was examined in five strains each of methicillin-susceptible *S. aureus* (MSSA) and MRSA. The frequencies of spontaneous single-step mutants resistant to fluoroquinolones from MSSA and MRSA are shown in Table 1. The frequencies of spontaneous single-step mutations in *S. aureus* selected by quinolones were generally low, regardless of the susceptibilities of the strains to methicillin, but the frequency of mutations in P-20 was relatively high. The frequency of spontaneous single-step mutations in P-20 selected by 0.78 μg of ciprofloxacin per ml was 2.5×10^{-6}, which was more than 1,600 times higher than the frequency of spontaneous mutations in 209P JC-1. Even at a concentration of 1.56 μg/ml, 19 spontaneous single-step mutants from P-20 were selected by ciprofloxacin (Table 2). The frequency of spontaneous single-step mutations in P-20 selected by 0.78 μg of ofloxacin per ml was 7.8×10^{-7}, which was more than 520 times higher than that in 209P JC-1 (Table 1). Sixteen spontaneous single-step mutants from P-20 selected by 0.78 μg of ofloxacin per ml were obtained (Table 2). The frequency of

TABLE 1. Frequencies of spontaneous single-step mutants resistant to quinolones in *S. aureus*

S. aureus strain	MIC (μg/ml)[a]					Mutation frequency (10^{-9})[a,b]			
	AT-4140	CPFX	OFLX	NFLX	DMPPC	AT-4140	CPFX	OFLX	NFLX
209P JC-1	0.05	0.2	0.39	0.39	1.56	<1.5	<1.5	<1.5	380
MS16008	0.025	0.2	0.2	0.78	1.56	<3.3	30	<3.3	37
MS16373	0.0125	0.2	0.2	0.78	1.56	<3.0	21	<3.0	3.0
MS16415	0.025	0.2	0.2	0.78	1.56	<5.3	37	16	11
MS16427	0.05	0.39	0.39	1.56	1.56	<5.3	100	<5.3	53
MS16023	0.05	0.39	0.39	1.56	>100	<5.2	31	<5.2	16
MS16086	0.05	0.39	0.39	1.56	>100	<6.2	<6.2	<6.2	6.2
P-5	0.05	0.39	0.39	1.56	>100	<4.3	43	<4.3	17
P-20	0.05	0.2	0.2	0.78	>100	<4.9	2,500	780	780
P-86	0.05	0.2	0.2	0.78	>100	<4.3	210	100	150

[a] Abbreviations: CPFX, ciprofloxacin; OFLX, ofloxacin; NFLX, norfloxacin; DMPPC, methicillin.
[b] Mutation frequencies were examined at a selective concentration of four times the MICs.

TABLE 2. Drug susceptibilities of spontaneous single-step, quinolone-resistant *S. aureus* mutants

Organism (no. of mutants)	MIC (μg/ml) for individual mutants[a]							
	AT-4140	CPFX	OFLX	NFLX	NA	NB	MINO	VCM
S. aureus 209P JC-1	0.05	0.2	0.39	0.39	100	0.1	0.1	0.39
Spontaneous mutants of 209P JC-1 selected by norfloxacin at 1.56 μg/ml (25)	0.2	0.78	1.56	3.13_3, 6.25_{22}	800	0.05_{18}, 0.1_7	0.05_1, 0.1_{24}	0.39
S. aureus P-20	0.05	0.2	0.2	0.78	100	0.1	0.1	0.78
Spontaneous mutants of P-20 selected by:								
Ciprofloxacin at 1.56 μg/ml (19)	0.1	3.13	1.56	12.5_1, 25_{18}	100_{15}, 200_4	0.05_7, 0.1_{12}	0.1	0.78
Ofloxacin at 0.78 μg/ml (16)	0.1	1.56_{10}, 3.13_6	0.78_5, 1.56_{11}	6.25_4, 12.5_7, 25_5	100_2, 200_{14}	0.05_1, 0.1_{12}, 0.2_3	0.1	0.78
Norfloxacin at 6.25 μg/ml (14)	0.1	1.56_3, 3.13_{11}	1.56	12.5_{13}, 25_1	200	0.1_{13}, 0.2_1	0.1	0.78

[a] Abbreviations: CPFX, ciprofloxacin; OFLX, ofloxacin; NFLX, norfloxacin; NA, nalidixic acid; NB, novobiocin; MINO, minocycline; VCM, vancomycin. Inferior numbers are the number of mutants with the indicated MIC.

spontaneous single-step mutations in P-20 selected by 3.13 μg of norfloxacin per ml was 7.8×10^{-7} (Table 1), and 14 spontaneous single-step mutants of P-20 were obtained (Table 2). However, no spontaneous single-step mutants from MSSA and MRSA were detected when selected by AT-4140, even at a concentration of four times the MIC (Table 1).

Drug susceptibilities of spontaneous single-step mutants. The drug susceptibilities of spontaneous single-step quinolone-resistant mutants from strains 209P JC-1 and P-20 were tested. As shown in Table 2, the spontaneous single-step mutants from 209P JC-1 selected by 1.56 μg of norfloxacin per ml were 8- to 16-fold less susceptible to norfloxacin and were 4- to 8-fold less susceptible to other quinolones, including AT-4140 and nalidixic acid. They were essentially unchanged from the parent strain 209P JC-1 in their susceptibilities to novobiocin, minocycline, or vancomycin, which are structurally unrelated to quinolones.

Nineteen spontaneous single-step mutants from P-20 selected by ciprofloxacin at a concentration of eight times the MIC (1.56 μg/ml) were 16-fold less susceptible to ciprofloxacin. The mutants were also 8- to 32-fold less susceptible to ofloxacin and norfloxacin, but they were only 1- to 2-fold less susceptible to AT-4140 and nalidixic acid. Similar results were obtained with the mutants from P-20 that were selected by ofloxacin and norfloxacin. No spontaneous single-step, quinolone-resistant mutants from P-20 selected by ciprofloxacin, ofloxacin, and norfloxacin were resistant to novobiocin, minocycline, or vancomycin.

Spontaneous double-step mutants selected by quinolones. The second-step mutation to quinolone resistance in P-20 was also examined. *S. aureus* P-20-C was a spontaneous single-step mutant of P-20 selected by ciprofloxacin at a concentration of eight times the MIC (1.56 μg/ml). It was 16-, 8-, and 32-fold more resistant to ciprofloxacin, ofloxacin, and norfloxacin, respectively, but only 2-fold more resistant to AT-4140 in comparison with P-20, its parent strain (see Table 4). The frequencies of spontaneous second-step mutation in P-20-C were low when selected at a concentration of four times the MIC: 4.1×10^{-8} for AT-4140, 1.0×10^{-8} for ciprofloxacin, 7.7×10^{-9} for ofloxacin, and 7.7×10^{-9} for norfloxacin (Table 3). These mutants were usually highly resistant to all quinolones tested. The MICs of AT-4140, ciprofloxacin, ofloxacin, and norfloxacin for the mutants selected by AT-4140 or ofloxacin were 6.25, 12.5, 12.5, and 50 μg/ml, respectively (Table 4). In contrast, one of four mutants selected by ciprofloxacin and all three mutants selected by norfloxacin showed different susceptibility profiles. They were highly resistant to ciprofloxacin, ofloxacin, and norfloxacin; but they were slightly less susceptible to AT-4140 than their parent strain P-20-C was. Spontaneous second-step, quinolone-resistant mutants of P-20 were still susceptible to novobiocin, minocycline, and vancomycin.

DISCUSSION

Clinical isolates of ciprofloxacin-resistant MRSA were divided into two diverse groups with respect to their susceptibilities to AT-4140: group 1, which were moderately or highly resistant to ciprofloxacin, ofloxacin, and norfloxacin but practically susceptible to AT-4140 (MICs, 0.05 to 0.39 μg/ml), and group 2, which were resistant to all fluoroquinolones tested, including AT-4140 (MICs, 1.56 to 25 μg/ml). The results demonstrate the presence of a difference in the susceptibility profiles between AT-4140 and existing fluoroquinolones in MRSA isolates.

The emergence of quinolone-resistant MRSA is probably a result of a selective pressure from quinolones that are used clinically against *S. aureus*. To investigate this hypothesis, we examined the frequencies of spontaneous quinolone-resistant mutations in *S. aureus*. The frequencies of spontaneous single-step quinolone-resistant mutations in *S. aureus* ranged from $<1.5 \times 10^{-9}$ to 2.5×10^{-6} when selected by ciprofloxacin, ofloxacin, or norfloxacin at a concentration of four times the MIC; and it was difficult to conclude whether methicillin resistance affected the frequency of quinolone-resistant mutations in *S. aureus* (Table 1). An interesting finding was that no spontaneous single-step, quinolone-resistant mutants were detected in any of the *S. aureus*

TABLE 3. Frequencies of spontaneous mutations to quinolone resistance in *S. aureus* P-20-C[a]

Compound	Selected MIC (concn [μg/ml])	Mutation frequency
AT-4140	4 × MIC (0.39)	4.1×10^{-8}
	8 × MIC (0.78)	1.3×10^{-8}
	16 × MIC (1.56)	7.7×10^{-9}
Ciprofloxacin	4 × MIC (12.5)	1.0×10^{-8}
	8 × MIC (25)	$<2.6 \times 10^{-9}$
Ofloxacin	4 × MIC (6.25)	7.7×10^{-9}
	8 × MIC (12.5)	$<2.6 \times 10^{-9}$
Norfloxacin	4 × MIC (100)	7.7×10^{-9}
	8 × MIC (200)	$<2.6 \times 10^{-9}$

[a] *S. aureus* P-20-C is a spontaneous single-step, quinolone-resistant mutant of P-20 that was selected by ciprofloxacin at a concentration of 1.56 μg/ml.

TABLE 4. Drug susceptibilities of spontaneous double-step quinolone-resistant mutants of *S. aureus* P-20

Organism (no. of mutants)	MIC (μg/ml) for individual mutants[a]							
	AT-4140	CPFX	OFLX	NFLX	NA	NB	MINO	VCM
S. aureus P-20	0.05	0.2	0.2	0.78	100	0.1	0.1	0.78
S. aureus P-20-C[b]	0.1	3.13	1.56	25	100	0.1	0.1	0.78
Spontaneous mutants of P-20-C selected by:								
AT-4140 at 1.56 μg/ml (3)	6.25	12.5	12.5	50	400	0.1	0.1	0.78
Ciprofloxacin at 12.5 μg/ml (4)	0.39_1, 6.25_3	25	6.25_1, 12.5_3	100	400_1, 800_3	0.2	0.1	1.56
Ofloxacin at 6.25 μg/ml (3)	6.25	12.5	12.5	50	400	0.1	0.1	1.56
Norfloxacin at 100 μg/ml (3)	0.2_1, 0.39_2	50_1, 100_2	12.5_1, 25_2	400_1, $>400_2$	400_2, 800_1	0.2	0.1	1.56

[a] See footnote *a* of Table 2.
[b] See footnote *a* of Table 3.

strains tested with AT-4140 at a concentration of four times the MIC (Table 1). Spontaneous single-step, quinolone-resistant mutants selected by ciprofloxacin, ofloxacin, or norfloxacin were resistant to all existing fluoroquinolones; but they were less resistant to AT-4140 (Table 2). Therefore, spontaneous single-step, quinolone-resistant mutants selected by ciprofloxacin, ofloxacin, or norfloxacin in vitro appeared to correspond to group 1 of the clinical isolates of ciprofloxacin-resistant MRSA.

In order to investigate how group 2 isolates emerge, we isolated spontaneous double-step, quinolone-resistant mutants from *S. aureus*. A clinical isolate of MRSA, P-20, was used for this study because it had the highest frequencies of quinolone-resistant mutations among the strains tested. First, a spontaneous quinolone-resistant mutant, termed P-20-C, was selected by ciprofloxacin, and then spontaneous second-step, quinolone-resistant mutants were selected from P-20-C by various quinolones by using higher selective concentrations. The frequencies of spontaneous second-step, quinolone-resistant mutations ranged from 7.7×10^{-9} to 4.1×10^{-8} when selected by AT-4140, ciprofloxacin, ofloxacin, or norfloxacin at concentrations of four times the MICs for P-20-C (Table 3). All spontaneous second-step, quinolone-resistant mutants selected by AT-4140 or ofloxacin and most mutants selected by ciprofloxacin were moderately or highly resistant to all quinolones tested, including AT-4140 (MIC, 6.25 μg/ml; Table 4). Therefore, they appeared to correspond to group 2 of the clinical isolates of ciprofloxacin-resistant MRSA. All spontaneous second-step, quinolone-resistant mutants selected by norfloxacin and one mutant selected by ciprofloxacin were moderately or highly resistant to ciprofloxacin, ofloxacin, and norfloxacin but were less resistant to AT-4140 (MICs, 0.2 to 0.39 μg/ml) and appeared to belong to group 1.

Spontaneous single-step mutants from *S. aureus* 209P JC-1 selected by norfloxacin were 4, 4, 4, 8 to 16, and 8 times more resistant to AT-4140, ciprofloxacin, ofloxacin, norfloxacin, and nalidixic acid, respectively, than their parent strain was (Table 2), suggesting that these mutants have mutations that affect their susceptibilities to all quinolones. Such a mutation may be located on the *gyr* genes, as *gyr* mutations in *Escherichia coli* affect its susceptibility to all quinolones, with few exceptions (3, 12, 21–23). In contrast, all spontaneous single-step mutants from *S. aureus* P-20 selected by ciprofloxacin, ofloxacin, or norfloxacin were 8 to 16, 4 to 8, and 8 to 32 times more resistant to ciprofloxacin, ofloxacin, and norfloxacin, respectively, than their parent strain was; but they were less resistant to AT-4140 and nalidixic acid (Table 2), suggesting that some structure of the *S. aureus* cell may change so that it affects the permeability to or the efflux of quinolones (3–5, 9, 12). The outer membrane of gram-negative rods has been well accepted as a barrier to penetration of antibacterial agents into the bacterial cell body (3, 15), but the *S. aureus* cell does not possess an outer membrane structure. The barrier function may exist somewhere in the *S. aureus* cell structure, probably in the cytoplasmic membrane. It is worth pointing out that AT-4140 (unpublished data) and nalidixic acid are more lipophilic than ciprofloxacin, ofloxacin, and norfloxacin (2). The results obtained in this study indicate that the mechanism of quinolone resistance in *S. aureus* may not be simple, and the mechanism of quinolone resistance in *S. aureus* remains to be elucidated.

AT-4140 was well absorbed when given orally, and its average peak level in plasma in six healthy volunteers who received a single oral dose of 200 mg was about 0.7 μg/ml and its elimination half-life in plasma was about 16 h (M. Kanamaru, M. Nakashima, T. Uematsu, and Y. Takikuchi, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1490, 1989). Further examination of this agent as a potent candidate for treatment of MRSA is warranted because of its potent activity against *S. aureus* (8, 11), its improved oral efficacy against systemic and urinary tract infections models with *S. aureus* in mice (11), the low frequency of AT-4140-resistant mutants in *S. aureus*, and its good pharmacokinetic properties in healthy volunteers.

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