

RWJ-54428 (MC-02,479), a New Cephalosporin with High Affinity for Penicillin-Binding Proteins, Including PBP 2a, and Stability to Staphylococcal Beta-Lactamases

Francois Malouin,[†] Johanne Blais, Suzanne Chamberland,[‡] Monica Hoang, Craig Park, Christin Chan, Kristina Mathias, Samia Hakem, Kelly Dupree, Eric Liu, Tien Nguyen, and Michael N. Dudley*

Essential Therapeutics, Inc., Mountain View, California 94043

Received 25 June 2002/Returned for modification 24 September 2002/Accepted 15 November 2002

RWJ-54428 (MC-02,479) is a new cephalosporin active against gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA). The potency of this new cephalosporin against MRSA is related to a high affinity for penicillin-binding protein 2a (PBP 2a), as assessed in a competition assay using biotinylated ampicillin as the reporter molecule. RWJ-54428 had high activity against MRSA strains COL and 67-0 (MIC of 1 µg/ml) and also showed affinity for PBP 2a, with a 50% inhibitory concentration (IC₅₀) of 0.7 µg/ml. RWJ-54428 also displayed excellent affinity for PBP 5 from *Enterococcus hirae* R40, with an IC₅₀ of 0.8 µg/ml and a MIC of 0.5 µg/ml. The affinity of RWJ-54428 for PBPs of β-lactam-susceptible *S. aureus* (MSSA), enterococci (*E. hirae*), and *Streptococcus pneumoniae* showed that the good affinity of RWJ-54428 for MRSA PBP 2a and *E. hirae* PBP 5 does not compromise its binding to susceptible PBPs. RWJ-54428 showed stability to hydrolysis by purified type A β-lactamase isolated from *S. aureus* PC1. In addition, RWJ-54428 displayed low MICs against strains of *S. aureus* bearing the four classes of staphylococcal β-lactamases, including β-lactamase hyperproducers. The frequency of isolation of resistant mutants to RWJ-54428 from MRSA strains was very low. In summary, RWJ-54428 has high affinity to multiple PBPs and is stable to β-lactamase, properties that may explain our inability to find resistance by standard methods. These data are consistent with its excellent activity against β-lactam-resistant gram-positive bacteria.

β-Lactam antibiotics exert their antibacterial effect through covalent interactions with penicillin-binding proteins (PBPs). PBPs are anchored in the bacterial cytoplasmic membrane and are involved in the last steps of cell wall peptidoglycan synthesis. Most bacterial species possess multiple PBPs, differing in enzymatic function, molecular weight, and affinity for β-lactam antibiotics. Although resistance to β-lactam antibiotics is often associated with production of β-lactamase, target alterations have been found to mediate high-level β-lactam resistance in staphylococci, enterococci, and pneumococci (38).

The genetic determinant of methicillin resistance in staphylococci has been identified as the *mec* DNA. This 30- to 50-kb region of foreign DNA is found only in methicillin-resistant staphylococci (39). This DNA region contains the gene *mecA* encoding PBP 2a, which has low affinity for β-lactam antibiotics (5–6).

Resistance to β-lactams among other gram-positive bacteria has been reported with increasing frequency. High-level ampicillin resistance is frequently observed in vancomycin-resistant *Enterococcus faecium* (24). This high-level resistance is mediated by overproduction of some PBPs and a further decrease in

the affinity of these enzymes for β-lactam antibiotics (13). Penicillin resistance can now be found in 25% of clinical isolates of *Streptococcus pneumoniae* and viridans group streptococci worldwide (18). Resistance to penicillins and cephalosporins in streptococci is mediated by target site mutations in PBPs (6, 13, 32, 35).

β-Lactamase enzymes are responsible for resistance to antimicrobial therapy (2). In bacteria, the genes coding for these enzymes can be found on the chromosome, on plasmids, or on transposable elements (17). Currently, more than 90% of *Staphylococcus aureus* isolates worldwide are resistant to penicillin (8, 29, 31). The resistance genes have spread from staphylococci, and in 1982, penicillinase genes were first reported in *Enterococcus faecalis* (25–26).

Four immunotypic variants of *S. aureus* β-lactamase, termed A, B, C, and D, have been identified and characterized (19, 41). *S. aureus* β-lactamases are penicillin-hydrolyzing enzymes inhibited by clavulanic acid and, as such, are part of group 2a of Bush's classification (2).

RWJ-54428 is a new cephalosporin with a broad gram-positive spectrum of activity, which includes methicillin-resistant *S. aureus* (MRSA), methicillin-resistant coagulase-negative staphylococci, enterococci, and pneumococci highly resistant to other β-lactam antibiotics (3). This paper summarizes studies that were performed to determine the affinity of RWJ-54428 for several PBPs of *Staphylococcus*, *Enterococcus*, and *Streptococcus* and the stability of RWJ-54428 to relevant β-lactamases.

(Part of this work was previously disclosed in poster presentations [S. Chamberland, C. Chan, J. Blais, K. M. Mathias, F. Malouin, and V. J. Lee, Abstr. 37th Intersci. Conf. Antimicrob.

* Corresponding author. Mailing address: Essential Therapeutics, Inc., 850 Maude Ave., Mountain View, CA 94043. Phone: (650) 428-3571. Fax: (650) 428-3534. E-mail: mdudley@etrx.com.

[†] Present address: Université de Sherbrooke, Faculté des sciences, département de biologie and CEVDM, Sherbrooke, Québec, Canada J1K 2R1.

[‡] Present address: Ulysses Pharmaceuticals, Eastman, Québec, Canada J0E 1P0.

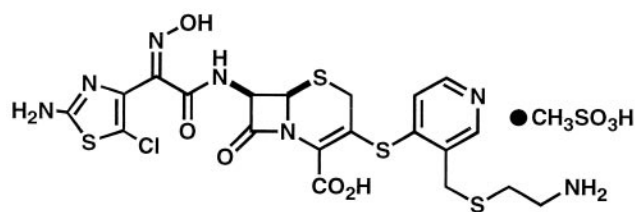


FIG. 1. RWJ-54428 (MC-02,479) chemical structure.

Agents Chemother., abstr. F-178, 1997; M. K Hoang, S. Chamberland, D. Griffith, and M. Dudley, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-016, 1998; T. Nguyen, E. Liu, F. Malouin, J. Blais, and S. Chamberland, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-017, 1998].

MATERIALS AND METHODS

Bacterial strains. Seven strains of methicillin-susceptible *S. aureus* (MSSA) representing four distinct staphylococcal β -lactamases (types A, B, C, and D) were studied (19). MRSA strains included COL, a β -lactamase negative homogeneously methicillin-resistant strain (14), strain 76, a β -lactamase-positive strain of MRSA (30), and a heterogeneously resistant strain of MRSA (strain 67-0) (14). Three strains of *E. faecalis* were also included: *E. faecalis* V583, a *vanB*-mediated vancomycin-resistant strain (10), was the β -lactamase negative control, whereas *E. faecalis* strain Beirut and strain HH22 produced type A β -lactamase. *Enterococcus hirae* R40, a derivative of *E. hirae* ATCC 9790 highly resistant to ampicillin, was used to study the affinity of RWJ-54428 for PBP 5 (11). Several genetically characterized strains of *S. pneumoniae* were used to study the affinity of RWJ-54428 for streptococcal PBPs (12): strains 43350 (serotype 23F), 2039 (serotype 6A), and 637 (serotype 23F). They were representative of the main European clones or had a serotype preferentially found in South Africa (36). The laboratory strain R6 was also used as a reference (1).

Antibiotics. RWJ-54428 was supplied as the mono-methanesulfonate salt. A stock solution of 2 mg of active RWJ-54428/ml was prepared in a 1:1 solution of dimethyl sulfoxide-sterile water. For the PBP binding assay, a stock solution of 5 mg of RWJ-54428/ml was prepared in sterile water. Commercially available antibiotics were obtained from various sources: ampicillin, cefaclor, cefamandole, cefazolin, cefotaxime, ceftazidime, cephalothin, cephaloridine, erythromycin, methicillin, nafcillin, oxacillin, penicillin G, and vancomycin were obtained from Sigma Chemical Co. Imipenem-cilastatin (Primaxin) was purchased from Merck. All antibiotics, except imipenem and erythromycin, were prepared at a concentration of 10 mg/ml in sterile water. Imipenem was prepared at a concentration of 5 mg/ml in sterile water. Erythromycin was prepared at a concentration of 5 mg/ml in ethanol. Stock solutions were aliquoted and kept frozen at -80°C . Each aliquot was rapidly thawed and only used once.

Antibiotic susceptibility testing. Susceptibility tests were performed using a broth microdilution assay according to NCCLS reference methods (28) in a final volume of 100 μl , using cation-adjusted Mueller-Hinton broth (CAMHB) (Difco). The culture medium was supplemented with 2% NaCl when testing β -lactam antibiotics against *S. aureus* isolates. The MICs of the drugs for streptococci were determined in CAMHB supplemented with 2.5% lysed horse blood (Remel).

Antibiotics were prepared at a concentration equivalent to two times the highest desired final concentration. Antibiotics were then diluted directly in the 96-well microtiter plates by serial twofold dilution by using a multichannel pipette. Microtiter plates were incubated for 24 h at 35°C and were read using a microtiter plate reader (Molecular Devices) at 650 nm as well as by visual observation using a microtiter plate reading mirror. Inoculum effect studies were carried out by performing susceptibility testing using both agar dilution and broth microdilution methods, as previously described (28), with modification of the starting inoculum.

Affinity for PBPs: preparation of bacterial membranes. Membrane-based PBP assays were used for measurement of RWJ-54428 affinity to MRSA PBP 2a and *E. hirae* PBP 5. MRSA strain *S. aureus* 67-0 was grown at 35°C in tryptic soy broth (Difco) containing 5 μg of nafcillin/ml, and the cell membrane fraction was extracted as described below. *E. hirae* R40 was grown at 35°C in brain heart infusion (Difco) without antibiotic. In both cases, exponentially growing cells

were harvested by centrifugation, washed, and stored at -80°C . Frozen cells were rapidly thawed, and initial cellular lysis was carried out in 50 mM Tris buffer (pH 7.5) containing lysostaphin (100 $\mu\text{g}/\text{ml}$) or lysozyme (500 $\mu\text{g}/\text{ml}$), 100 mM NaCl, 5 mM MgCl_2 , 1 mM phenylmethylsulfonyl fluoride, and 20 μg of DNase I and RNase A per ml at 35°C for 1 h before cells were mechanically disrupted using a French pressure cell. Unbroken cells were removed by low-speed centrifugation. Membranes were separated by differential ultracentrifugation from the cell extract and suspended in 10 mM Tris-HCl (pH 7) to a final concentration of 10 mg of protein/ml (4, 11).

Competition assays. PBPs were labeled using a biotinylated ampicillin (BIO-AMP) according to the method described by Dargis and Malouin (7). The reporter molecule, BIO-AMP, was also prepared as previously described (7). To selectively label PBP 2a from staphylococcal membrane preparations, samples were first incubated with 100 μg of clavulanic acid/ml for 10 min at 35°C to saturate high-affinity PBPs (PBPs 1, 2, and 3). The extremely low affinity of clavulanate for MRSA PBP 2a relative to that for the other PBPs was previously reported by Chambers and Sachdeva (4). The relative binding affinity of β -lactams for PBP 2a was then assayed, in a competition assay, by adding increasing concentrations of the test compound to aliquots of the reaction mixture containing 20 μg of membrane preparation for 15 min at 35°C prior to the addition of BIO-AMP (20 μM final concentration) for an additional 15 min. Clavulanic acid was omitted in experiments determining the binding of the test compounds to any other PBPs. Samples were then boiled for 4 min in electrophoretic loading buffer containing sodium dodecyl sulfate (SDS), and proteins were separated by electrophoresis on an SDS-polyacrylamide discontinuous gel system (5% stacking and 8% separating gels). After electrophoresis, proteins were transferred onto nitrocellulose membranes. Electrophoretic PBP profiles were detected on blots by using an ECL chemiluminescence reagent system (Amersham), on the basis of the interaction of BIO-AMP-PBP complexes and an avidin-peroxidase conjugate (Bio-Rad Laboratories) (7). The concentration of the test β -lactam needed to block 50% of the subsequent binding of BIO-AMP to each PBP of interest (50% inhibitory concentration [IC_{50}]) was determined by scanning the PBP profiles obtained on an ECL hyperfilm (Amersham) or on a chemiluminescence screen using a Molecular Imager (Bio-Rad). In some cases, the binding of the test β -lactam to the entire PBP set was evaluated by measuring the reduction of the overall chemiluminescence signal compared to that of the control PBP profile obtained in the absence of the test β -lactam and was expressed as the percentage of inhibition of BIO-AMP binding to PBPs.

Whole-cell assays. PBP competition assays for MSSA and *S. pneumoniae* strains were performed with intact bacterial cells. For each concentration of test compound, an aliquot of freshly grown cells (equivalent to 1 ml of a bacterial suspension with an A_{600} of 0.4) was substituted for the cell membrane preparation in the competition assay described above. After the 15-min incubation period, the labeling reaction was quickly stopped by the addition of ice-cold unlabeled penicillin G (1 mg/ml) and cells were collected by centrifugation. Cells were suspended in a lysis buffer (described above) for 15 min at 37°C , and cell debris were collected by centrifugation in a tabletop Avanti centrifuge (Beckman) with the F3606 rotor at maximum speed for 20 min. Supernatants were removed, electrophoretic loading buffer was added to the membrane pellet, and samples were boiled for 4 min before separation of proteins on SDS-polyacrylamide gel electrophoresis and transfer onto nitrocellulose membrane as described above.

β -Lactamase preparation. Extracellular β -lactamase was purified from *S. aureus* PC1 producing type A β -lactamase. Staphylococcal β -lactamase extract was prepared by the method described by Kernodle et al. (20–21).

β -Lactamase stability assay. Analysis of the kinetics of hydrolysis by purified PC1 β -lactamase was performed as follows: a concentration of 100 μM antibiotic was used in 0.1 M sodium phosphate buffer, pH 7.0. Reactions were followed at wavelengths which corresponded to the maximal change in absorbance between the unhydrolyzed substrate and the hydrolyzed product for each β -lactam, as follows: cephaloridine (254 nm), cefamandole (269 nm), cefazolin (272 nm), cefaclor (264 nm), penicillin G (232 nm), ampicillin (235 nm), nitrocefin (486 nm), and RWJ-54428 (290 nm). Selection of the wavelength to study the β -lactamase stability of RWJ-54428 was based on the results of validation assays, which demonstrated the disappearance of RWJ-54428 following the addition of sodium hydroxide. The assay was performed in a 1-cm quartz cuvette at room temperature, using a Perkin-Elmer Lambda Bio UV/VIS spectrometer. Only the initial linear part of the curve was used in calculations of the β -lactamase hydrolysis rate. Results were expressed as the relative rate of hydrolysis (RRH), and cephaloridine hydrolysis was set at 100%. The β -lactamase stability assay was repeated three times using three different preparations of enzymes. The results presented below in Table 4 are representative of one of the assays. The compound ranking order was similar in each of the assays performed.

TABLE 1. Binding affinity of RWJ-54428 and known β -lactam antibiotics toward a selection of PBPs from β -lactam-susceptible and -resistant gram-positive bacteria

Bacteria	Antibiotic	MIC ($\mu\text{g/ml}$)	IC ₅₀ for indicated PBP in competition assays ($\mu\text{g/ml}$)					
			1	2	2a	3	4	5
MRSA 67-0	RWJ-54428	1	— ^a	—	0.7	—	—	—
	Ampicillin	16	—	—	23	—	—	—
	Imipenem	32	—	—	30	—	—	—
	Penicillin G	16	—	—	48	—	—	—
	Cefazolin	256	—	—	238	—	—	—
	Oxacillin	64	—	—	272	—	—	—
	Cefotaxime	128	—	—	363	—	—	—
	Cephalothin	128	—	—	363	—	—	—
	Nafcillin	256	—	—	385	—	—	—
Methicillin	512	—	—	587	—	—	—	
MSSA ATCC 29213	RWJ-54428	0.25	0.2	0.2	NA ^b	0.1	—	—
	Cefaclor	0.5	0.3	>8	NA ^b	<0.03	—	—
	Methicillin	1	0.6	6.6	NA ^b	—	—	—
	Nafcillin	0.5	0.3	0.4	NA ^b	0.3	—	—
	Imipenem	<0.06	<0.01	0.03	NA ^b	0.04	—	—
<i>E. hirae</i> R40	RWJ-54428	0.5	—	—	NA ^b	<0.5	<0.5	0.8
	Imipenem	32	—	—	NA ^b	<0.5	<0.5	8.6
<i>S. pneumoniae</i> R6 ^c	RWJ-54428	<0.06	0.1	0.2	0.05	0.2	0.1	—
	Penicillin G	<0.06	0.04	0.04	0.03	0.2	0.03	—

^a —, not determined.

^b NA, not applicable.

^c For *S. pneumoniae* R6, PBPs, 1a, 1b, 2x, 2a, 2b, and 3 (columns from left to right) were evaluated.

Resistance selection. Selection of resistance to RWJ-54428 was studied in six strains of MSSA and two strains of MRSA. For each *S. aureus* strain, a single isolated colony was grown overnight in 8 ml of MHB. A volume of 250 μl of each bacterial suspension (undiluted and diluted) was then applied onto solid medium containing various concentrations of RWJ-54428 by using the Autoplate 4000 (Spiral Biotech, Inc.). Inocula were determined by applying 10- μl drops of 10-fold dilutions onto tryptic soy agar (TSA; Difco) plates. The plates were incubated at 35°C for 48 h. CFU were counted after 24 and 48 h of incubation at 35°C. Ratios of the number of colonies on the drug-containing plates to that on the control plates were calculated as the in vitro frequency of isolation of CFU for each strain tested. If colonies were isolated from drug-containing agar, their susceptibility to RWJ-54428 was compared to that of the parent strain by using the spiral gradient endpoint method (Spiral Biotech, Inc.).

RESULTS

Affinity of PBPs. The results of competition assays for PBPs with RWJ-54428 and imipenem are shown in Fig. 2 and 3, and a comparison of IC₅₀ values is shown in Table 1. RWJ-54428 had a higher affinity for MRSA PBP 2a (IC₅₀ = 0.7 $\mu\text{g/ml}$) than imipenem (IC₅₀ = 30 $\mu\text{g/ml}$). There was a generally good

correlation between β -lactam MICs and affinities to MRSA PBP 2a (Table 1).

RWJ-54428 has high affinity for the well-characterized *E. hirae* PBP 5. The IC₅₀ values for *E. hirae* PBP 5 were 0.8 and 8.6 $\mu\text{g/ml}$ for RWJ-54428 and imipenem, respectively (Table 1).

RWJ-54428 has high affinity for multiple PBP targets from both β -lactam-susceptible and -resistant gram-positive strains. RWJ-54428 showed IC₅₀ values comparable to those of other traditional β -lactams, such as nafcillin for MSSA, imipenem for *E. hirae*, and penicillin G for *S. pneumoniae*, in the sense that the low MIC could be associated with the IC₅₀ for one or more PBPs. As opposed to that seen against MRSA PBP 2a, imipenem was very potent and showed very low IC₅₀ values for MSSA PBPs (Table 1 and Fig. 3). Table 2 compares the binding of RWJ-54428 to that of penicillin G for PBPs from penicillin-susceptible, penicillin-intermediate, and penicillin-resistant isolates of *S. pneumoniae*. By measuring the residual

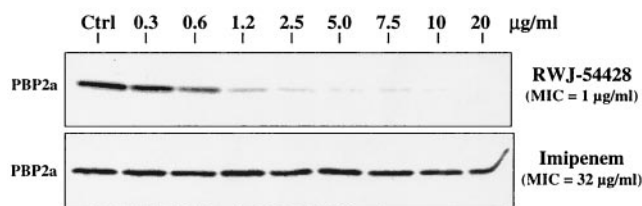


FIG. 2. PBP competition assay demonstrating the affinity of RWJ-54428 toward PBP 2a of MRSA strain 67-0 in comparison to that with imipenem.

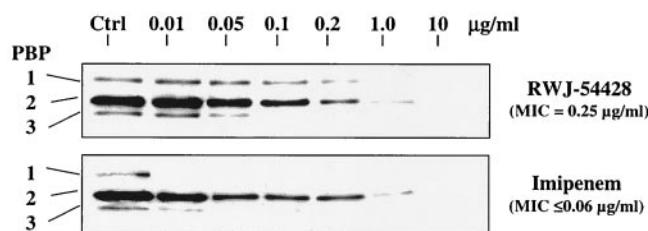
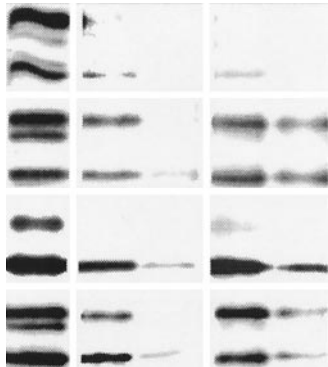


FIG. 3. Whole-cell PBP competition assay demonstrating the affinity of RWJ-54428 toward the PBPs of MSSA strain ATCC 29213 in comparison to that with imipenem.

TABLE 2. Affinity of RWJ-54428 toward PBPs of selected *S. pneumoniae* strains compared with that of penicillin G (Pen G)

Strain	PBP profiles				Inhibition of BIO-AMP binding (%)				MIC ($\mu\text{g/ml}$)		
	Ctrl	RWJ-54428 ($\mu\text{g/ml}$)		PenG ($\mu\text{g/ml}$)		RWJ-54428 ($\mu\text{g/ml}$)		Pen G ($\mu\text{g/ml}$)		RWJ-54428	Pen G
		0.2	2	0.2	2	0.2	2	0.2	2		
R6						89	100	97	100	≤ 0.06	≤ 0.06
43350 (23F)						68	98	45	70	0.25	1
2039 (6A)						66	96	43	82	0.5	2
637 (23F)						66	97	45	87	0.25	4

chemiluminescence signal of BIO-AMP in the presence of RWJ-54428 compared to that measured in the control PBP profiles, it was shown that the overall binding of RWJ-54428 for *S. pneumoniae* PBPs was equivalent to or surpassed that of penicillin G in PBP competition assays (Table 2).

Stability of RWJ-54428 to β -lactamases. Comparison of the stability of RWJ-54428 to staphylococcal and enterococcal β -lactamases is shown in Table 3. There was no more than a twofold difference in the RWJ-54428 MIC between MSSA ATCC 25923, a β -lactamase-negative strain, and MSSA strains producing type A, B, and D β -lactamases. A fourfold-higher MIC was observed in MSSA PC1, which overproduces type A β -lactamase, and MSSA V137, which produces a type C β -lactamase. For these two strains, MICs of RWJ-54428 were 0.5 $\mu\text{g/ml}$. In MRSA strains, there was only a twofold difference in the RWJ-54428 MIC between a β -lactamase-positive strain (MRSA 76) and a β -lactamase-negative strain (MRSA COL). In contrast, β -lactamase production in MSSA was associated with a 32- to 4,096-fold-higher penicillin G MIC, 16- to 512-fold-higher ampicillin MIC, and 4- to 8-fold-higher cefamandole and cefaclor MICs compared to those for the β -lactamase-negative control strain (ATCC 25923).

The hydrolysis of RWJ-54428 and other β -lactam antibiotics related to cephaloridine by purified PC1 β -lactamase of *S. aureus* was studied (Table 4). The hydrolysis of RWJ-54428 by type A β -lactamase was low, indicating stability of this new cephalosporin to this enzyme. PC1 staphylococcal preferentially hydrolyzes penicillins, as the RRHs for penicillin and ampicillin were 30- to 55-fold greater than those observed for RWJ-54428. RWJ-54428 has rates of hydrolysis that are comparable to those of cefazolin.

In vitro inoculum effect. The effect of inoculum size on the in vitro activity of RWJ-54428 against various strains of *S. aureus* was determined by using broth microdilution and agar dilution methods. In the agar dilution assays (Table 5), there was no significant increase in the MIC of RWJ-54428 for strains with inocula ranging from 5×10^4 to 5×10^7 CFU/spot. Only twofold variations in susceptibility to RWJ-54428 were

observed for the *S. aureus* strains tested, including β -lactamase-positive strains. In contrast, the activity of penicillin G, which is highly susceptible to degradation by staphylococcal β -lactamases, against β -lactamase-positive strains was dramatically reduced (by 16- to 128-fold) with a 100-fold larger inoculum. There was a two- to eightfold increase in MICs of cefamandole and cefaclor when tested against the β -lactamase-positive MSSA strains at an inoculum 1,000-fold larger (i.e., $>10^7$ CFU/spot).

In broth microdilution assays, the susceptibilities of MRSA and MSSA strains to RWJ-54428 were reduced by two- to eightfold only when the inoculum was equal or greater than 500 times ($\sim 10^8$ CFU/ml) the NCCLS recommended inoculum (data not shown). The eightfold increase in MIC was observed in a β -lactamase-negative strain, indicating that β -lactamase does not contribute to this observation. In contrast, strong inoculum effects (2- to 256-fold increases in MICs) were noted for commercially available antibiotics such as cefamandole, cefaclor, cefazolin, cefotaxime, ampicillin, penicillin G, and vancomycin with inocula 50 times the NCCLS recommended inoculum size (data not shown).

In studies conducted with RWJ-54428 in the broth microdilution assay, samples from sub-MIC wells at higher inocula were streaked onto antibiotic-free TSA plates and incubated aerobically at 35°C for 24 h. When colonies were isolated, their susceptibility to RWJ-54428 was determined. There was no change in susceptibility for these isolated colonies compared to the parent strains.

Selection of resistance. Attempts to select and obtain *S. aureus* isolates resistant to RWJ-54428 were unsuccessful. In the instances where growth was observed and colonies were picked from the selective media, endpoint susceptibility tests showed that these isolates were as susceptible as the parental strains to RWJ-54428, with MICs varying by no more than twofold. This was the case whether the test strain used in these frequency-of-resistance studies was MRSA, MSSA, β -lactamase-positive or -negative, or when the inoculum was as large as 10^9 CFU/ml.

TABLE 3. Susceptibility of staphylococci and enterococci producing β -lactamases to RWJ-54428 and other β -lactam antibiotics

Bacteria	β -Lactamase ^a		MIC (μ g/ml) ^b				
	Type	Level	RWJ	PEN G	AMP	FAM	CEC
MSSA ATCC 25923	Neg. ^c	0	0.125	0.015	0.06	0.25	0.125
MSSA ATCC 29213	A	0.006	0.25	0.5	1	0.25	0.5
MSSA NCTC 9789	A	0.088	0.25	32	16	1	1
MSSA PC1	A	0.201	0.5	64	32	1	2
MSSA 22260	B	0.024	0.125	4	4	2	1
MSSA V137	C	0.034	0.5	8	8	2	1
MSSA FAR19	D	0.030	0.25	4	2	1	1
MRSA COL	Neg. ^c	0	1	16	16	>8	>8
MRSA 76	Pos. ^d	NA ^e	2	64	>32	>8	>8
<i>E. faecalis</i> VanB	Neg. ^c	0	0.125	ND ^f	1	ND ^f	ND ^f
<i>E. faecalis</i> Beirut	A	NA ^e	\leq 0.0625	ND ^f	2	ND ^f	ND ^f
<i>E. faecalis</i> HH22	A	NA ^e	\leq 0.0625	ND ^f	8	ND ^f	ND ^f

^a From Kernodle et al. (12, 13). The activity was reported as micromoles of nitrocefin degraded per minute per cell mass after induction.

^b RWJ, RWJ-54428; PEN G, penicillin G; AMP, ampicillin; FAM, cefamandole; CEC, cefaclor.

^c Neg., β -lactamase-negative strain.

^d Pos., β -lactamase-positive strain for which the enzyme type was not determined.

^e NA, not available.

^f ND, not done.

DISCUSSION

The quest for a β -lactam that inhibits MRSA PBP 2a has now been conducted for several years. Many studies have aimed at characterizing structural and enzymatic features of this low-affinity PBP target (22, 33, 37). RWJ-54428 is a new parenteral cephalosporin with potent activity against multi-drug-resistant gram-positive bacteria, including staphylococci, enterococci, and pneumococci highly resistant to other β -lactam antibiotics (3). The potent activities of RWJ-54428 against β -lactam-resistant staphylococci and enterococci were correlated with high affinities for staphylococcal PBP 2a and PBP 5 from enterococci, both major determinants of resistance to β -lactam antibiotics. The enhanced binding of RWJ-54428 to MRSA PBP 2a is correlated to a pharmacophore that is structurally distinct from that of other reported anti-MRSA β -lactams (16). The pharmacophore of RWJ-54428 also provides binding affinity to *E. hirae* PBP 5, which shares some common features with MRSA PBP 2a and *E. faecium* PBP 5 (9, 40). The high affinity of RWJ-54428 to PBP 5 is consistent with previously reported susceptibility data against 100 isolates of ampicillin-resistant *E. faecium* showing that RWJ-54428 has activity against these isolates (RWJ-54428 MIC at which 50% of isolates are inhibited [MIC_{50}] of 4 μ g/ml; MIC_{90} of 8 μ g/ml) (3). However, not all reported anti-MRSA β -lactams have affinity for the enterococcal PBP 5 and, consequently, inhibitory activities against that species may greatly vary (15, 23, 27).

Although we demonstrated the potent in vitro activity against a large number of clinical isolates of methicillin-resistant and methicillin-susceptible staphylococci (*S. aureus* and coagulase-negative staphylococci), enterococci, and pneumococci (3), we needed to verify that the high-affinity PBP 2a/PBP 5 pharmacophore of RWJ-54428 did not impede binding to other crucial PBP targets found in β -lactam-susceptible organisms. The affinity of RWJ-54428 for PBPs of *S. aureus*, *E. hirae*, and *S. pneumoniae* was high. RWJ-54428 binds well to both resistant and susceptible PBPs from staphylococci, enterococci, and pneumococci. The low MICs of RWJ-54428 for these β -lactam-susceptible gram-positive bacteria were associated with low IC_{50} values for one or more PBPs. Studies with penicillin-susceptible, -intermediate, and -resistant pneumococci demonstrated that RWJ-54428 also has high affinity for penicillin-resistant and -susceptible PBPs in pneumococci.

The β -lactamase stability of RWJ-54428 was demonstrated by comparing the susceptibilities of various strains of *S. aureus* producing several types and amounts of β -lactamases (A, B, C, and D). The stability of RWJ-54428 to staphylococcal β -lactamase was further substantiated with biochemical studies. Partly purified PC1 enzyme was used to determine the RRHs for RWJ-54428 and several other β -lactam antibiotics. The RRH of RWJ-54428 by type A staphylococcal β -lactamase was low compared to those of other β -lactams, indicating increased stability of this new cephalosporin to this enzyme. RRHs for reference compounds were in accordance with previously published data: penicillin G RRH, 1,869; ampicillin RRH, 675; cefamandole RRH, 6.4; cefazolin RRH, 30.6; and nitrocefin RRH, 2,034 (41).

Significant inoculum effects with *S. aureus* have been reported for antibiotics susceptible to hydrolysis by β -lactamases, but not for β -lactamase-stable β -lactam antibiotics (34). In agar dilution assays and microdilution broth assays, increasing inoculum size did not significantly reduce the activity of RWJ-54428 against the MSSA and MRSA strains studied, regardless of the production of β -lactamase or the type of enzyme produced by these strains (type A, B, C, or D).

Numerous efforts to isolate spontaneous resistant mutants of *S. aureus* by passage on media containing various concentrations of RWJ-54428 were unsuccessful. In these studies, the production of β -lactamase in various strains of *S. aureus* did not provide more opportunities to select resistance to RWJ-54428. Both the high affinity of RWJ-54428 for multiple PBPs and its stability to staphylococcal β -lactamases are attributes that may reduce the rate of isolation of resistance to this new cephalosporin in *S. aureus*.

In summary, RWJ-54428 is a new cephalosporin with potent activity against staphylococci, including MRSA. The enhanced activity of RWJ-54428 against MRSA is associated with high affinity for PBP 2a, the primary determinant of methicillin resistance in *Staphylococcus* spp. (6). This corroborates the potent antistaphylococcal activity of RWJ-54428 that was previously reported against a large population of strains demon-

TABLE 4. RRH of RWJ-54428 and other β -lactam antibiotics by the purified PC1 β -lactamase of *S. aureus*

Antibiotic	RRH ^a	Strain PC1 ^b MIC (μ g/ml)
Cephaloridine	100	0.5
RWJ-54428	33	0.5
Penicillin G	1,791	64
Ampicillin	987	32
Cefamandole	40	1
Cefazolin	35	1
Cefaclor	213	2
Nitrocefin	1,821	ND ^c

^a Expressed as a percentage of the RRH of cephaloridine.

^b MICs were determined by a broth microdilution method.

^c ND, not done.

TABLE 5. Effect of inoculum size on activity of RWJ-54428 against MRSA and MSSA strains with various types and levels of production of β -lactamase, as determined using the agar dilution assay

<i>S. aureus</i> strain	β -Lactamase ^a		Antibiotic	MIC (μ g/ml) at CFU/spot inoculum size:			
	Type	Level		5×10^4	5×10^5	5×10^6	5×10^7
MSSA							
ATCC 25923	Neg. ^b	0	RWJ-54428	0.125	0.125	0.25	0.25
			Cefamandole	0.25	0.5	0.5	0.5
			Cefaclor	1	1	2	2
			Penicillin G	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03
ATCC 29213	A	0.006	RWJ-54428	0.25	0.25	0.25	0.25
			Cefamandole	0.5	0.5	1	1
			Cefaclor	1	2	2	8
			Penicillin G	0.125	0.25	2	32
NCTC 9789	A	0.088	RWJ-54428	0.25	0.25	0.25	0.5
			Cefamandole	0.5	1	1	4
			Cefaclor	2	4	8	>8
			Penicillin G	1	8	>64	>64
			Vancomycin	0.5	1	2	16
22260	B	0.24	RWJ-54428	0.25	0.25	0.25	0.25
			Cefamandole	0.5	1	2	4
			Cefaclor	2	2	4	8
			Penicillin G	0.5	2	32	>64
V137	C	0.034	RWJ-54428	0.25	0.25	0.5	0.5
			Cefamandole	1	1	2	8
			Cefaclor	2	4	8	>8
			Penicillin G	1	8	64	>64
FAR19	D	0.03	RWJ-54428	0.25	0.25	0.25	0.25
			Cefamandole	0.5	0.5	1	2
			Cefaclor	1	2	4	8
			Penicillin G	0.25	1	32	>64
MRSA							
COL	Neg. ^b	NA ^d	RWJ-54428	1	1	1	1
			Cefamandole	>8	>8	>8	>8
			Penicillin G	16	32	32	32
			Vancomycin	2	2	2	4
76	Pos. ^c	NA	RWJ-54428	1	1	1	1
			Cefamandole	>8	>8	>8	>8
			Penicillin G	32	64	>64	>64
			Vancomycin	1	1	2	8

^a From Kernodle et al. (20, 21). The activity was reported as micromoles of nitrocefin degraded per minute per cell mass after induction.

^b Neg., β -lactamase-negative strain.

^c Pos., β -lactamase-positive strain for which the enzyme type was not determined.

^d NA, not available.

strating MIC₉₀s of 0.5, 2, and 2 μ g/ml against MSSA, MRSA, and MR coagulase-negative staphylococci, respectively (3). RWJ-54428 also binds to multiple PBPs from gram-positive bacteria. These properties combined with a stability to staphylococcal β -lactamase render RWJ-54428 a potent agent for resistant gram-positive strains and species of clinical importance.

ACKNOWLEDGMENTS

We thank B. Murray, R. Fontana, R. Hakenbeck, and D. Kernodle for the enterococci, pneumococci, and MSSA strains with distinct β -lactamases.

The work described herein was conducted as part of a research collaboration with the R.W. Johnson Pharmaceutical Institute.

REFERENCES

1. Avery, O. T., C. M. MacLeod, and M. McCarty. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *J. Exp. Med.* **79**:137-158.
2. Bush, K., G. A. Jacoby, and A. A. Medeiros. 1995. A functional classification scheme for β -lactamases and its correlation with molecular structure. *Antimicrob. Agents Chemother.* **39**:1211-1233.
3. Chamberland, S., J. Blais, M. Hoang, C. Dinh, D. Cotter, E. Bond, C. Gannon, C. Park, F. Malouin, and M. N. Dudley. 2001. In vitro activity of RWJ-54428 (MC-02,479) against multiresistant gram-positive bacteria. *Antimicrob. Agents Chemother.* **45**:1422-1430.
4. Chambers, H. F., and M. Sachdeva. 1990. Binding of β -lactam antibiotics to penicillin-binding proteins in methicillin-resistant *Staphylococcus aureus*. *J. Infect. Dis.* **161**:1170-1176.
5. Chambers, H. F. 1997. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *Clin. Microbiol. Rev.* **10**:781-791.
6. Chambers, H. F. 1999. Penicillin-binding protein-mediated resistance in pneumococci and staphylococci. *J. Infect. Dis.* **179**(Suppl. 2):S353-S359.

7. Dargis, M., and F. Malouin. 1994. Use of biotinylated β -lactams and chemiluminescence for study and purification of PBPs in bacteria. *Antimicrob. Agents Chemother.* **38**:973–980.
8. Doern, G. V., R. N. Jones, M. A. Pfaller, K. C. Kugler, and M. L. Beach. 1999. Bacterial pathogens isolated from patients with skin and soft tissue infections: frequency of occurrence and antimicrobial susceptibility patterns from the SENTRY antimicrobial surveillance program (United States and Canada, 1997). *Diagn. Microbiol. Infect. Dis.* **34**:65–72.
9. El Kharroubi, A., P. Jacques, G. Piras, J. Van Beeumen, J. Coyette, and J. M. Ghuysen. 1991. The *Enterococcus hirae* R40 penicillin-binding protein 5 and the methicillin-resistant *Staphylococcus aureus* penicillin-binding protein 2' are similar. *Biochem. J.* **280**:463–469.
10. Evers, S., D. F. Sahn, and P. Courvalin. 1993. The vanB gene of vancomycin-resistant *Enterococcus faecalis* V583 is structurally related to genes encoding D-alanine ligases and glycopeptide-resistance proteins VanA and VanC. *Gene* **124**:143–144.
11. Fontana, R., R. Cerini, P. Longoni, A. Grossato, and P. Canepari. 1983. Identification of a streptococcal PBP that reacts very slowly with penicillin. *J. Bacteriol.* **155**:1343–1350.
12. Hakenbeck, R., T. Briese, L. Chalkley, H. Ellerbrok, R. Kalliokoski, C. Latorre, M. Leinonen, and C. Martin. 1991. Antigenic variation of penicillin-binding proteins from penicillin-resistant clinical strains of *Streptococcus pneumoniae*. *J. Infect. Dis.* **164**:313–319.
13. Hakenbeck, R. 1998. Resistant penicillin-binding proteins. *Cell. Mol. Life Sci.* **54**:332–340.
14. Hartman, B. J., and A. Tomasz. 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *J. Bacteriol.* **158**:513–516.
15. Hebeisen, P., I. Heinze-Krauss, P. Angehrn, P. Hohl, M. G. P. Page, and R. L. Then. 2001. In vitro and in vivo properties of Ro 63-9141, a novel broad-spectrum cephalosporin with activity against methicillin-resistant staphylococci. *Antimicrob. Agents Chemother.* **45**:825–836.
16. Hecker, S. J., T. W. Glinka, A. Cho, Z. J. Zhang, M. E. Price, S. Chamberland, D. Griffith, and V. J. Lee. 2000. Discovery of RWJ-54428 (MC-02,479), a new cephalosporin active against resistant gram-positive bacteria. *J. Antibiot.* **53**:1272–1281.
17. Jacoby, G. A., and G. L. Archer. 1991. New mechanisms of bacterial resistance to antimicrobial agents. *N. Engl. J. Med.* **324**:601–612.
18. Jones, R. N., and W. R. Wilson. 1998. Epidemiology, laboratory detection, and therapy of penicillin-resistant streptococcal infections. *Diagn. Microbiol. Infect. Dis.* **31**:453–459.
19. Kernodle, D. S., C. W. Stratton, L. W. McMurray, J. R. Chipley, and P. A. McGraw. 1989. Differentiation of β -lactamase variants of *Staphylococcus aureus* by substrate hydrolysis profiles. *J. Infect. Dis.* **159**:103–108.
20. Kernodle, D. S., D. J. Zygmunt, P. A. McGraw, and J. R. Chipley. 1990. Purification of *Staphylococcus aureus* β -lactamases by using sequential cation-exchange and affinity chromatography. *Antimicrob. Agents Chemother.* **34**:2177–2183.
21. Kernodle, D. S., P. A. McGraw, C. W. Stratton, and A. B. Kaiser. 1990. Use of extracts versus whole-cell bacterial suspensions in the identification of *Staphylococcus aureus* β -lactamase variants. *Antimicrob. Agents Chemother.* **34**:420–425.
22. Lu, W.-P., Y. Sun, M. D. Bauer, S. Paule, P. M. Koenigs, and W. G. Kraft. 1999. Penicillin-binding protein 2a from methicillin-resistant *Staphylococcus aureus*: kinetic characterization of its interactions with β -lactams using electrospray mass spectrometry. *Biochemistry* **38**:6537–6546.
23. Matsumoto, M., H. Tamaoka, H. Ishikawa, and M. Kikuchi. 1998. In vitro and in vivo antibacterial activities of OPC-20011, a novel parenteral broad-spectrum 2-oxaisocephem antibiotic. *Antimicrob. Agents Chemother.* **42**:2943–2949.
24. Murray, B. E. 1998. Diversity among multidrug-resistant enterococci. *Emerg. Infect. Dis.* **4**:37–47.
25. Murray, B. E., and B. Mederski-Samoraj. 1983. Transferable β -lactamase: a new mechanism for *in vitro* penicillin resistance in *Streptococcus faecalis*. *J. Clin. Investig.* **72**:1168–1171.
26. Murray, B. E., K. V. Singh, S. M. Markowitz, H. A. Lopardo, J. E. Patterson, M. J. Zervos, E. Rubeglio, G. M. Caputo, R. Nasnas, L. S. Moore, E. S. Wong, and G. Weinstock. 1991. Evidence for clonal spread of a single strain of β -lactamase-producing *Enterococcus (Streptococcus) faecalis* to six hospitals in five states. *J. Infect. Dis.* **163**:780–785.
27. Nagano, R., K. Shibata, Y. Adachi, H. Imamura, T. Hashizume, and H. Morishima. 2000. In vitro activities of novel *trans*-3,5-disubstituted pyrrolidinylthio-1 β -methylcarbapenems with potent activities against methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **44**:489–495.
28. National Committee for Clinical Laboratory Standards. 1997. Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed., vol. 17, no. 2. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.
29. O'Brien, T. K., and The International Survey of Antibiotic Resistance Group. 1986. Resistance to antibiotics at medical centers in different parts of the world. *J. Antimicrob. Chemother.* **18**(Suppl. C):243–253.
30. Peacock, J. E., F. J. Marsik, and R. P. Wenzel. 1980. Methicillin-resistant *Staphylococcus aureus*: introduction and spread within a hospital. *Ann. Intern. Med.* **93**:526–532.
31. Pfaller, M. A., R. N. Jones, G. V. Doern, and K. Kugler. 1998. Bacterial pathogens isolated from patients with bloodstream infection: frequencies of occurrence and antimicrobial susceptibility patterns from the SENTRY antimicrobial surveillance program (United States and Canada, 1997). *Antimicrob. Agents Chemother.* **42**:1762–1770.
32. Reichmann, P., A. Konig, A. Marton, and R. Hakenbeck. 1996. Penicillin-binding proteins as resistance determinants in clinical isolates of *Streptococcus pneumoniae*. *Microb. Drug Resist.* **2**:177–181.
33. Roychoudhury, S., J. E. Dotzlaw, S. Ghag, and W.-K. Yeh. 1994. Purification, properties, and kinetics of enzymatic acylation with β -lactams of soluble penicillin-binding protein 2a, a major factor in methicillin-resistant *Staphylococcus aureus*. *J. Biol. Chem.* **269**:12067–12073.
34. Sabath, L. D., C. Garner, C. Wilcox, and M. Finland. 1975. Effect on inoculum and of beta-lactamase on the anti-staphylococcal activity of thirteen penicillins and cephalosporins. *Antimicrob. Agents Chemother.* **8**:344–349.
35. Severin, A., et al. 1997. Abnormal physiological properties and altered cell wall composition in *Streptococcus pneumoniae* grown in the presence of clavulanic acid. *Antimicrob. Agents Chemother.* **41**:504–510.
36. Sibold, C., J. Wang, J. Henrichsen, and R. Hakenbeck. 1992. Genetic relationships of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated on different continents. *Infect. Immun.* **60**:4119–4126.
37. Sun, Y., M. D. Bauer, and W. Lu. 1998. Identification of the active site serine of penicillin-binding protein 2a from methicillin-resistant *Staphylococcus aureus* by electrospray mass spectrometry. *J. Mass Spectrom.* **33**:1009–1016.
38. Tomasz, A., and R. Munoz. 1995. β -Lactam antibiotic resistance in gram-positive bacterial pathogens of the upper respiratory tract: a brief overview of mechanisms. *Microb. Drug Resist.* **1**:103–109.
39. Wu, S. W., H. deLencastre, and A. Tomasz. 2001. Recruitment of the *mecA* gene homologue of *Staphylococcus sciuri* into a resistant determinant and expression of the resistant phenotype in *Staphylococcus aureus*. *J. Bacteriol.* **183**:2417–2424.
40. Zorzi, W., X. Y. Zhou, O. Dardenne, J. Lamotte, D. Raze, J. Pierre, L. Gutmann, and J. Coyette. 1996. Structure of the low-affinity penicillin-binding protein 5 PBP5fm in wild-type and highly penicillin-resistant strains of *Enterococcus faecium*. *J. Bacteriol.* **178**:4948–4957.
41. Zygmunt, D. J., C. W. Stratton, and D. S. Kernodle. 1992. Characterization of four β -lactamases produced by *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **36**:440–445.