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Dual Targeting of Topoisomerase IV and Gyrase To Reduce Mutant Selection: Direct Testing of the Paradigm by Using WCK-1734, a New Fluoroquinolone, and Ciprofloxacin

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Quinolones that act equally against DNA gyrase and topoisomerase IV are a desirable modality to decrease the selection of resistant strains. We first determined by genetic and biochemical studies in *Staphylococcus aureus* that the primary target enzyme of WCK-1734, a new quinolone, was DNA gyrase. A single mutation in gyrase, but not topoisomerase IV, caused a two- to fourfold increase in the MIC. Studies with purified topoisomerase IV and gyrase from *S. aureus* also showed that gyrase was more sensitive than topoisomerase IV to WCK-1734 (50% inhibitory concentration, 1.25 and 2.5 to 5.0 μg/ml, respectively; 50% stimulation of cleavage complex formation, 0.62 and 2.5 to 5.0 μg/ml, respectively). To test the effect of balanced activity of quinolones against the two target enzymes, we measured the frequency of selection of mutants with ciprofloxacin (which targets topoisomerase IV) and WCK-1734 alone and in combination. With the combination of ciprofloxacin and WCK-1734, each at its MIC, the ratio of frequency of mutants selected was significantly lower than that with each drug alone at two times their respective MICs. We further characterized resistant strains selected with the combination of ciprofloxacin and WCK-1734 and found evidence to suggest the existence of novel mutational mechanisms for low-level quinolone resistance. By use of a combination of differentially targeting quinolones, this study provides novel data in direct support of the paradigm for dual targeting of quinolone action and reduced development of resistance.

Type II DNA topoisomerases, DNA gyrase and topoisomerase IV (TopoIV), are essential enzymes that regulate changes in DNA topology by catalyzing the concerted breakage and rejoining of DNA strands during normal cellular growth. They catalyze the relaxation of supercoiled DNA, catenation and decatenation of DNA rings, and knotting and unknotting of duplex DNA (37). DNA gyrase is unique in its ability to catalyze negative DNA supercoiling. Gyrase and TopoIV are heterotetramers of $GyrA_2GyrB_2$ and $ParC_2ParE_2$, respectively. GyrA and ParC are the subunits responsible for DNA binding and the cleavage and religation reaction, and GyrB and ParE are responsible for ATP binding and hydrolysis (22).

Quinolones act by forming ternary complexes with DNA and either DNA gyrase or TopoIV, thereby blocking DNA replication and triggering events leading to cell death (5, 12). Quinolone resistance occurs stepwise by mutations in the two topoisomerase target enzymes, with the first mutation generally occurring in the more sensitive enzyme (13). From the first-step mutants second-step double mutants can then be selected with resistance mutations in the second target enzyme, thereby conferring a high-level resistance phenotype (1, 7, 21). If the original sensitivities of both DNA gyrase and TopoIV were the same (i.e., dual targeting), no single mutational alteration in either enzyme would result in an increase in the MIC (11, 25). Resistance would require, instead, concurrent alteration in both enzymes. Because spontaneous double mutations are rare genetic events (occurring at a frequency of 10^{-14} to 10^{-16} for fluoroquinolones), it has been postulated that the use of fluoroquinolones with dual activity could limit the selection of fluoroquinolone resistance in wild-type bacteria (33, 39).

Recently reported quinolones that manifested MICs lower than those of older quinolones against gram-positive bacteria have also selected for resistant bacteria at a very low frequency at concentrations at or near the MIC and with mutations appearing in novel locations outside the classical quinolone-resistance-determining region (QRDR) (18, 29). The low frequency of mutants selected was ascribed to their dual-targeting properties as manifested by the modest and similar effect on the MIC of either *parC* or *gyrA* mutations (whereas mutations in both target enzymes caused substantial increases in the MIC) and by the ability to select for mutants only at or close to the MIC. This argument in support of the paradigm that dual-targeting drugs impede selection of resistant mutants is, however, circumstantial. In addition, a notable property of the resistant mutants selected by these new drugs is that mutations conferring resistance in *parC*, *parE*, *gyrA*, or *gyrB* are located beyond the classical QRDRs. It is unknown if this pattern implies a unique mechanism of interaction of the drugs with their targets that could also play a role in the selection of mutants or if it is secondary to the equality or near equality in activity against the two target enzymes that these particular quinolones exhibit.

We recently investigated a novel quinolone, WCK-1734, for which the primary target is gyrase. We report here the effects of genetically defined mutants of *Staphylococcus aureus* on the activity of WCK-1734 and characterize single-step mutants selected by WCK-1734 to determine the primary target of WCK-1734 in *S. aureus*. We describe, in addition, a more recent

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype[a]	Source or reference
S. aureus strains		
ISP794	8325 *pig-131*	34
SS1	8325 *pig-131 gyrB142* (Ile102Ser, Arg144Lys) *gyrA* (Ser84Leu)	2
MT5224c4	8325 *nov (gyrB142) hisG15 pig-131 parC542* (Ser80Phe)	36
EN1252a	8325 *nov* (*gyrB142*) *hisG15 pig-131 parC542 gyrA* Ω *1051* (Erm) Nov^+	2
Cip-B	ISP794 *parC* (D79A)	This study; spontaneous Cip^r
Cip-N	ISP794 *parC* (A116E)	This study; spontaneous Cip^r
1734-G	ISP794 *parE* (insertion 82G83) *gyrA*(S84L)	This study; spontaneous WCK-1734^r
1734-J	ISP794 *gyrA* (G82D)	This study; spontaneous WCK-1734^r
1734-P	ISP794 *parC* (R626H) *gyrA*(S84L)	This study; spontaneous WCK-1734^r
1734-S	ISP794 *gyrA* (G82D)	This study; spontaneous WCK-1734^r
1734/cip-P	ISP794 *parE* (G30R)	This study; spontaneous WCK-1734/Cip^r
1734/cip-T	ISP794 *parC* (F18L)	This study; spontaneous WCK-1734/Cip^r
1734-J-AE-15	ISP794 *gyrA* (G82D)	This study; allelic exchange mutant
1734-J-AE-18	ISP794 *gyrA* (G82D)	This study; allelic exchange mutant
E. coli strains		
DH5α	F^- ϕ80d*lacZ* Δ*M15* Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 phoA hsdR17* (r_K^- m_K^-) *supE44*λ^-*thi-1 gyrA96 relA1*	GIBCO-BRL
TOP10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 recA1 ara*Δ139 Δ(*ara-leu*)7697 *galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Plasmids		
pBAD/Thio-TOPO	4.5-kb linearized expression vector, Ap^r	Invitrogen
pTrcHisB-gyrB	*gyrB* cloned into cloning vector pTrcHisB	Invitrogen
pCL52.1	8,100-bp plasmid containing the replicon of pGB2, Sp^r (*E. coli*) and temperature-sensitive replicon of pE194, Tc^r	23

[a] Abbreviations: WCK-1734^r, WCK-1734 resistant; Cip^r, ciprofloxacin resistant; Ap^r, ampicillin resistant; Sp^r, spectinomycin resistant; Tc^r, tetracycline resistant.

method for overexpression and purification of wild-type *S. aureus* GyrA and report *S. aureus* TopoIV and gyrase inhibitory assays and cleavage complex formation assays by WCK-1734 in direct comparison with ciprofloxacin. The definition of gyrase as the target for WCK-1734 and TopoIV as the target of ciprofloxacin then allowed us to assess the effect of dual targeting on the pattern and mechanism of resistance in *S. aureus* by comparing the effects of the drugs used alone and in combination for selection of resistant mutants. These data provide additional direct evidence in support of the dual-targeting paradigm.

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MATERIALS AND METHODS

Bacterial strains, vectors, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Beckton Dickinson, Sparks, MD). For mutant selection *S. aureus* strains were grown in brain heart infusion (BHI) (Beckton Dickinson, Sparks, MD) broth, and for MIC testing they were grown in Mueller-Hinton broth (Beckton Dickinson, Sparks, MD). All strains were grown at 37°C, except for *E. coli* with the plasmid pSAGA1, which was grown at 25°C for induction with arabinose. Ampicillin was used at a concentration of 100 μg/ml.

Drug susceptibility determinations. WCK-1734 was kindly provided by Wockhardt Research Centre, Aurangabad, India, and ciprofloxacin was kindly provided by the Bayer Corporation (West Haven, CT). Novobiocin and all other chemicals, unless stated otherwise, were purchased from Sigma Chemical Co. (St. Louis, Mo.). MICs were determined by the agar dilution method (24). The MIC was the lowest concentration of antibiotic that yielded no visible growth after incubation at 37°C for 24 h. MICs were determined with and without reserpine (20 μg/ml) to screen for changes in efflux pump expression (32).

Studies of the inhibitory interactions of the two quinolones were performed by a checkerboard broth microdilution technique (6). Combinations of WCK-1734 and ciprofloxacin were tested at concentrations of 0.00025 to 0.032 and 0.002 to 0.25 μg/ml, respectively. Inocula of ca. 10^5 CFU/ml were applied, plates were incubated overnight at 37°C, and MICs were read as recommended by NCCLS (24). The fractional inhibitory concentration (FIC) index was calculated by adding the FICs (MIC of drug A in combination with drug B divided by MIC of drug A alone) of WCK-1734 and ciprofloxacin. An FIC index of ≤0.5 was defined as synergy, an FIC index of >0.5 to 4.0 was defined as indifferent, and an FIC index of >4.0 was defined as antagonistic. Checkerboard test results represented the average of duplicate experiments.

Frequency of selection of mutants. Mutants were selected by plating appropriate dilutions of overnight cultures of *S. aureus* ISP794 on BHI agar containing WCK-1734, ciprofloxacin, or both at increasing concentrations at or above the MIC of each drug up to the limit at which no mutants could be selected (4). Plating of dilutions of these same cultures on drug-free BHI agar was used to determine the number of CFU plated on the selection plates. When needed for selection with WCK-1734 and the combination of WCK-1734 and ciprofloxacin, large (150 mm by 15 mm) petri dishes were used to plate ca. 10^{11} CFU. Selection plates were incubated at 37°C. The frequency of selection of resistant mutants was calculated as the ratio of the number of resistant colonies at 48 h to the number of CFU plated. Selected colonies of various sizes were purified on plates containing the same concentration of drug. Mutants were then maintained at −70°C in BHI broth containing 10% glycerol.

Sequence analysis. Chromosomal DNA from various mutants of *S. aureus* ISP794 was isolated using the Easy-DNA kit (Invitrogen, Carlsbad, Calif.) after lysing the cells with lysostaphin (Ambi, Lawrence, N.Y.) at 0.1 mg/ml in phosphate-buffered saline and was used as a template for PCRs. PCR amplifications for the entirety of *parC*, *parE*, *gyrA*, and *gyrB* and for the promoter regions of *parE* and *gyrB* were performed using Platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, Calif.). The primers used are shown in Table 2.

PCR amplification of the entire structural genes of *parC*, *parE*, *gyrA*, and *gyrB* involved two steps: five cycles followed by 30 cycles at annealing temperatures of 54.5°C and 48.0°C, respectively, both steps with an extension time of 180 s for *parC* and *gyrA* and 120 s for *parE* and *gyrB*. For the amplification of the promoter regions of *parE* and *gyrB*, the annealing temperatures were 46.0°C and 36.0°C, respectively, with extension times of 52 and 60 s, respectively, both for 35 cycles.

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TABLE 2. Primers used in this study

Gene	Forward primer	Reverse primer
Structural genes		
parC	5′AAATGATCAATTTGATGAGGAGGAA	5′TTATCATTTAATTTCGTGATTGCATATAG
parE	5′GTTTGCAGGAGGCGAAATCA	5′CCTAAAACATCTTCAAGTGATAAATCTTG
gyrA	5′GCTGAATTACCTCAATCAAGAATAAATG	5′TTATTATTCTTCATCTGATGATTGTTGTATATC
gyrB	5′GTGACTGCATTGTCAGATGTAAACAAC	5′TTATTAGAAGTCTAAGTTTGCATAAACTGC
Promoter region genes		
parE	5′CGCCGATAAGATAACTTAGTAG	5′GCTCTTGACGCTCTTTACCAC
gyrB	5′TAGACGATGTACTCAGTGAAT	5′GTGGCATATCCTGAGTTATAT
gyrB-gyrA with allelic exchange	5′TTAGTAGAATTCTTCTATCGATTTATGAGACCG	5′CCTTCAGGATCCATTAACTCAGCAATTGAAATAT

DNA sequencing of the PCR products was performed using Taq DyeDeoxy Terminator (Applied Biosystems) with the ABI 3700 PRISM automated sequencer (Massachusetts General Hospital core facility). All selected mutants were first sequenced for at least the first 500 bases of the *parC* and *gyrA* genes. All genetically defined mutants selected with WCK-1734 and those selected with the combination of WCK-1734 and ciprofloxacin were sequenced for the entirety of the *parC*, *parE*, *gyrA*, and *gyrB* genes.

Cloning of *S. aureus parC*, *parE*, *gyrA*, and *gyrB* genes. Cloning of the complete genes of *S. aureus* ISP794 *parC*, *parE*, and *gyrB* into pTrcHisC, pTrcHisA, and pTrcHisB, respectively, and overexpression and purification of the corresponding proteins ParC, ParE, and GyrB were performed as previously described (18). For cloning of *gyrA*, a 2,663-bp fragment containing the entire *gyrA* structural gene was amplified using a forward primer with the 5′ nucleotide at position 2155 in the sequence published by Ito et al. (20) (GenBank accession number D10489) and a reverse primer with the 5′ nucleotide at position 4818 (Table 2). PCR amplification was carried out with genomic DNA from strain ISP794 using Platinum *Pfx* DNA Polymerase (Invitrogen, Carlsbad, Calif.) according to the manufacturer's recommendations. The PCR amplification of *gyrA* was as noted above, except that a 3′ A-overhang was added to the amplified product by incubating it with 1 unit of *Taq* DNA polymerase (New England Biolabs, Beverly, MA) at 72°C for 10 min. The final PCR product was cloned directly into the pBAD/Thio-TOPO vector (Invitrogen, Carlsbad, Calif.), adding coding sequences for an N-terminal histidine patch containing thioredoxin fusion protein, to generate the pSAGA1 vector, which was electroporated into *E. coli* DH5α. Electrotransformants were selected on ampicillin-containing agar. We further screened by restriction digestion analysis for the constructs with the correct insert orientation. The insert DNA was then sequenced to confirm the absence of polymerase-generated or other mutations.

Allelic exchange. For the allelic exchange experiment, a fragment of the *gyrB-gyrA* tandem genes (GenBank accession number D10489) from strain 1734-J was amplified with the upstream and downstream primers (Table 2) containing engineered EcoRI and BamHI sites, respectively, to amplify the region between nucleotides 1746 to 2784. The annealing temperature was 50°C, and the extension time was 63 s for this PCR. Following gel extraction with the QIAquick gel extraction kit (QIAGEN, Valencia, CA), the PCR product was ligated into the EcoRI and BamHI sites of pCL52.1, a thermosensitive shuttle vector, and the recombinant plasmid was electroporated into *E. coli* DH5α. The insert was then transformed into *S. aureus* RN4220 and subsequently to *S. aureus* ISP794, as previously described (26). The insert from the final transformation was sequenced to confirm that no additional mutations were introduced by the DNA polymerase. Allelic exchange was performed as previously described (23). The resulting colonies were screened for susceptibility to tetracycline at a concentration of 5 μg/ml and reduced susceptibility to WCK-1734 at a concentration of 0.008 μg/ml.

Protein overexpression and purification. pSAGA1 was chemically transformed into TOP10 *E. coli* (Invitrogen, Carlsbad, Calif.). Twenty-five milliliters of an overnight culture of TOP10 *E. coli* with pSAGA1 in LB broth containing 100 μg of ampicillin per ml were used to inoculate 500 ml of fresh antibiotic-containing broth. The cells were grown to mid-logarithmic phase by vigorous shaking at 37°C and induced by the addition of arabinose at a final concentration of 0.2 mg/ml with further incubation for 3 h at 25°C. The cell pellet was harvested by centrifugation at 4°C, resuspended in 25 ml of 50 mM Tris-Cl (pH 8.0)–150 mM NaCl–10% (vol/vol) glycerol, rapidly frozen, and stored at −70°C until protein isolation. Cells were lysed by incubating on ice with lysozyme (0.1 mg/ml), Brij-58 (0.12%), and a protease inhibitor cocktail (Roche) for 1 h, followed by further incubation with DNase at a final concentration of 5 μg/ml. Following centrifugation, the supernatant was applied to a nickel iminodiacetic acid column (GE Healthcare/Amersham Biosciences), washed with 10 ml of 20 mM Tris-Cl (pH-8.0)–400 mM NaCl, and eluted in 25 mM imidazole–20 mM Tris-Cl (pH 8.0)–100 mM NaCl at room temperature. The subsequent steps were carried out at 4°C. The eluate was incubated overnight with enterokinase (Novagen, Cambridge, MA), titrated to yield 50% cleavage of the thioredoxin-gyraseA fusion protein by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Following cleavage, recombinant enterokinase was removed according to the manufacturer's recommendations, and the solution of cleaved protein was dialyzed three times against 50 mM Tris-Cl (pH 7.5)–100 mM KCl–10% (vol/vol) glycerol and reapplied on a nickel iminodiacetic acid column to adsorb any remaining noncleaved fusion protein. The cleaved protein solution was concentrated in a dialysis bag (Spectra/Por MWCO 3,500; Spectrum, Rancho Dominguez, CA) using dry polyethylene glycol compound (molecular weight, 15,000 to 20,000), and applied to a size-exclusion column (HiPrep 16/60 Sephacryl S-200 HR; GE Healthcare/Amersham Biosciences, Piscataway, N.J.) equilibrated in 50 mM Tris-Cl (pH 7.5)–100 mM KCl–10%(vol/vol) glycerol–2 mM dithiothreitol (DTT)–1 mM EDTA. The protein fractions were examined by SDS-polyacrylamide gel electrophoresis, and those containing GyrA protein were pooled and concentrated again using polyethylene glycol compound, dialyzed against the same buffer, rapidly frozen, and stored at −70°C. GyrA protein purified in this manner yielded a subunit-specific activity (in the presence of excess GyrB) of 2×10^5 U/mg, substantially higher than that previously reported (18), presumably due to the avoidance of the need for the resolubilization of the subunit from inclusion bodies.

Topoisomerase catalytic and DNA cleavage assays. Two units of each of the subunits, GyrA plus GyrB or ParC plus ParE, were preincubated together for 30 min on ice to reconstitute gyrase or TopoIV holoenzymes, respectively. DNA supercoiling activity was assayed with relaxed pBR322 DNA (0.5 μg; John Innes Enterprises, Norwich, United Kingdom) as a substrate. The reaction mixture (20 μl) contained 75 mM Tris-HCl (pH 7.5), 7.5 mM $MgCl_2$, 7.5 mM DTT, 2 mM ATP, 75 μg of bovine serum albumin per ml, 30 mM KCl, 250 mM potassium glutamate, 2 μg of tRNA, and various concentrations of quinolones. The reaction was carried out at 30°C for 30 min.

TopoIV decatenation activity was assayed using 105 ng of kinetoplast DNA (kDNA; from *Crithidia fasciculata*) (TopoGEN, Inc., Columbus, Ohio) as a substrate. The reaction mixture (20 μl) contained 50 mM Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 5 mM DTT, 50 μg/ml bovine serum albumin, 250 mM potassium glutamate, 1 mM ATP, and quinolones as specified. The reaction was carried out at 37°C for 30 min.

DNA cleavage assays were carried out as for the catalytic assays, except that ATP was omitted and the DNA substrate used was negatively supercoiled pBR322 DNA (John Innes Enterprises, Norwich, United Kingdom) at a concentration of 16 μg/ml. After 30 min, 2 μl of both SDS (5%) and proteinase K (1 mg/ml) was added, and the incubation was continued at 45°C for an additional 30 min.

Reactions were stopped by adding a mixture of EDTA (final concentration, 50 mM), bromphenol blue, and glycerol. All 20 μl of each reaction mixture was loaded onto a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and run at 3.5 V/cm for 14 to 16 h. Gels were stained with 0.6 μg of ethidium bromide per ml for 60 min, destained in water, and visualized under UV light.

Statistical analysis. The *t* test was used for comparisons of ratios of frequencies of mutant selection.

RESULTS

Activities of ciprofloxacin and WCK-1734 individually against genetically defined mutants. We first determined the

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MICs of WCK-1734 against genetically defined mutants of *S. aureus* and compared those with the MICs of ciprofloxacin (Table 3). The MICs of ciprofloxacin determined in this study were identical to or within twofold of those previously reported (18). A *gyrA* mutation leading to a Ser84Leu change produced no change in the MIC of ciprofloxacin, but a Ser80Phe mutation in TopoIV caused an eightfold increase in the MIC of ciprofloxacin, a pattern that is consistent with its primary action targeting TopoIV. A strain with mutations in both *gyrA* and *parC* genes was highly resistant to ciprofloxacin, with a 64-fold increase in the MIC in comparison to the wild-type strain. WCK-1734 was 32-fold more active than ciprofloxacin against wild-type *S. aureus* ISP794. WCK-1734 also differed in the resistance pattern for the same mutant set. The Ser80Phe mutation in ParC did not change the MIC of WCK-1734, whereas the Ser84Leu mutation in GyrA caused a consistent twofold increase in MIC. These results suggested that the primary target for WCK-1734 was gyrase.

Activity of the ciprofloxacin-WCK-1734 combination. Because we planned to combine ciprofloxacin and WCK-1734 to select for resistant mutants, we tested the interaction between the two drugs. According to the checkerboard titration method, the FIC index of the ciprofloxacin–WCK-1734 combination was 1.5 to 2.0, indicating an indifferent interaction between the two drugs.

Characterization of single-step mutants selected by each drug alone. To ascertain the primary cellular target, we selected mutants with WCK-1734 or ciprofloxacin. Mutants selected by ciprofloxacin at two- to fivefold the MIC maintained the wild-type MIC for WCK-1734 and manifested *parC* mutations located within the QRDR (Table 4) that have previously been shown to confer resistance to ciprofloxacin (25, 36).

We characterized four independent mutants selected with WCK-1734 (Table 4). Two mutants, 1734-P and 1734-G, selected at two- to fourfold the MIC (0.016 to 0.032 μg/ml) had an increased MIC of 8- to 16-fold for WCK-1734 and fourfold for ciprofloxacin. Both had a Ser84Leu mutation in GyrA. This mutation, as previously determined for the *gyrA* mutant SS1, was sufficient to confer low-level resistance to WCK-1734 and does not affect susceptibility to ciprofloxacin. Therefore, to account for the additional resistance phenotype of these mutants, we sequenced the entirety of the *gyrA*, *gyrB*, *parC*, and *parE* genes and found novel mutations: Arg626His in ParC in mutant 1734-P and a glycine insertion between amino acids 82 and 83 in ParE in mutant 1734-G. The MIC of novobiocin for both mutants was 0.16 to 0.32 μg/ml, which was unchanged from that of ISP794. Two other mutants, 1734-S and 1734-J, selected with WCK-1734 at two to three times its MIC, had a two- to fourfold increased MIC of WCK-1734 and a twofold increased or an unchanged MIC of ciprofloxacin. Sequencing the entirety of *gyrA*, *gyrB*, *parC*, and *parE* identified a single novel mutation within the QRDR of GyrA, Gly82Asp, in both the 1734-S and 1734-J mutants. The MICs of WCK-1734 for the four mutants did not decrease with the addition of reserpine (20 μg/ml). Thus, in each of the four characterized mutants selected with WCK-1734, a mutation in *gyrA* was found, and in two of the four mutants a *gyrA* mutation was the only target mutation found, suggesting that gyrase is the primary target of WCK-1734, in contrast to ciprofloxacin, which targets TopoIV.

TABLE 3. Activity of WCK-1734 and ciprofloxacin against genetically defined mutants of *S. aureus*[a]

Strain	Mutation	MIC (μg/ml)	
		WCK-1734	Ciprofloxacin[a]
ISP794	Wild-type (parent)	0.008	0.25
MT5224c4	*parC* (Ser80Phe)	0.008	2.0
SS1	*gyrA* (Ser84Leu)	0.016	0.25
EN1252a	*parC* (Ser80Phe) *gyrA* (Ser84Leu)	0.25	16.0

[a] Determined by agar dilution assay.

Confirmation of the role of novel mutations in resistance by allelic exchange. An allelic exchange experiment was performed for the novel Gly82Asp GyrA mutation found in mutant 1734-J. After cells were grown at permissive temperature for excision of the plasmid pCL52.1, they were screened for susceptibility to tetracycline and resistance to WCK-1734. For mutant 1734-J, the MICs of WCK-1734 (0.032 μg/ml) and ciprofloxacin (0.25 μg/ml) for the original (1734-J) and two allelic exchange mutants (1734-J-AE-15 and 1734-J-AE-18) were identical, indicating that the GyrA Gly82Asp mutation was responsible for the fourfold increase in the MIC. DNA sequencing confirmed the presence of *gyrA* (Gly82Asp) in the alleles exchanged. The *gyrA* mutations present in mutants 1734-G and 1734-P were identical to those in the genetically defined mutant SS1 and presumably contributed a similar twofold increase in the MIC of WCK-1734. Additional *parE* or *parC* mutations in these mutants together with the *gyrA* mutation may have accounted for the higher level of WCK-1734 resistance (16-fold increase for 1734-G and eightfold increase for 1734-P) in these mutants. No mutants with either *parC* or *parE* mutations alone were found.

Comparative activities of WCK-1734 and ciprofloxacin against purified TopoIV and gyrase. Quinolone inhibition of TopoIV activity was measured as inhibition of decatenation of kDNA. Ciprofloxacin was two- to fourfold more active than WCK-1734 in reducing the intensity of kDNA minicircles by half (50 inhibitory concentration [IC_{50}]), with IC_{50}s for ciprofloxacin and WCK-1734 of 1.25 to 2.5 μg/ml and 2.5 to 5 μg/ml, respectively (Table 5).

To assay quinolone inhibition of gyrase, we determined the extent of inhibition of gyrase-mediated negative supercoiling of relaxed pBR322 DNA. In contrast to its relative antagonism of the catalytic activity of TopoIV, WCK-1734 was fourfold more potent than ciprofloxacin in inhibiting the supercoiling activity of gyrase, with IC_{50}s for WCK-1734 and ciprofloxacin of 1.25 μg/ml and 5 μg/ml, respectively.

To compare further the relative target preferences of WCK-1734 and ciprofloxacin, we used the cleavage-complex (CC) formation assay. WCK-1734 was 64-fold more potent than ciprofloxacin in stimulating half-maximal intensity of linear DNA cleavage complex formation (CC_{50}) with gyrase, whereas it was two- to fourfold less potent in promoting TopoIV-mediated CC formation (Table 5). Thus, in vitro data show that *S. aureus* gyrase is more sensitive to WCK-1734 than to ciprofloxacin, whereas the reverse is true for TopoIV. These results concur with the genetic studies that the primary enzyme target of ciprofloxacin was TopoIV, whereas that for WCK-1734 was gyrase.

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TABLE 4. Characteristics of single-step mutants of *S. aureus* ISP794 selected with ciprofloxacin, WCK-1734, or a combination of ciprofloxacin and WCK-1734

Strain	Selecting drug	Selecting drug concn	MIC (μg/ml)[a]			Mutation(s) in:			
			Cip	WCK-1734	WCK-1734/cip	ParC	ParE	GyrA	GyrB
ISP794			0.25	0.008	0.004/0.125				
Cip-B	Ciprofloxacin	0.5	2	0.008	0.004/0.125–0.008/0.25	D79A	None	None	None
Cip-N	Ciprofloxacin	1.25	2	0.008	0.008/0.25	A116E	None	None	None
1734-G	WCK-1734	0.032	1	0.125	0.032/1.0	None	Insert 82G83	S84L	None
1734-J	WCK-1734	0.024	0.25–0.5	0.032	0.008/0.25–0.016/0.5	None	None	G82D	None
1734-P	WCK-1734	0.016	1	0.064	0.016/0.5	R626H	None	S84L	None
1734-S	WCK-1734	0.016	0.25	0.016	0.004/0.125–0.008/0.5	None	None	G82D	None
1734/cip-A	WCK-1734/ciprofloxacin	0.008/0.25	0.25–0.5	0.008–0.016	0.008/0.25	None	None	None	None
1734/cip-P	WCK-1734/ciprofloxacin	0.008/0.25	0.25	0.008	0.008/0.25	None	G30R	None	None
1734/cip-T	WCK-1734/ciprofloxacin	0.012/0.375[b]	2	0.008	0.012/0.375	F18L	None	None	None
1734/cip-W	WCK-1734/ciprofloxacin	0.012/0.375	0.5	0.008	0.012/0.375	None	None	None	None
1734/cip-Y	WCK-1734/ciprofloxacin	0.012/0.375	0.25–0.5	0.008–0.016	0.012/0.375–0.016/0.5	None	None	None	None

[a] Determined by agar dilution.
[b] Drug concentrations between twofold agar dilution plates.

Because we planned to use WCK-1734 and ciprofloxacin together in whole-cell preparations, we tested for potential interactions between the two quinolones that might affect their relative target preferences. The CC assay is thought to reflect more closely the relevant intracellular action of quinolones than do assays of inhibition of catalytic activity (28), and for this reason we chose to study possible drug interactions in the CC assay. We measured cleavage complex stimulation of one drug, with the other drug added at half of its CC_{50}. In our experience, at this concentration the stimulation of CC formation was negligible. We postulated that if the two drugs were indifferent at the enzyme level, the CC_{50} for one drug in the presence of the other drug should not change by more than twofold from the CC_{50} of the one drug alone, because of the minimal stimulation for CCs by the first-added drug. When ciprofloxacin was added at 10 μg/ml, the CC_{50} of WCK-1734 for gyrase changed from 1.25 to 0.62 μg/ml. Similarly, when WCK-1734 was added, the ciprofloxacin CC_{50} for TopoIV changed by twofold or less, from 1.25 to 2.5 μg/ml to 1.25 μg/ml. Therefore, in in vitro assays, when WCK-1734 and ciprofloxacin are combined, they do not appear to interact in a way that might alter their differentially selective effects in interacting with their distinct targets.

Frequency of selection of mutants. The range of frequencies of selection of single-step resistant mutants of wild-type strain ISP794 with ciprofloxacin were similar to those previously published (15), and mutants could be selected at up to fivefold the MIC (Table 6). WCK-1734, at the same relative MICs as ciprofloxacin, selected for mutants at a frequency range that was somewhat lower but overlapped with that of ciprofloxacin, and mutants could be selected with WCK-1734 at a concentration of up to threefold the MIC. The frequency of selection of mutants for WCK-1734 at threefold the MIC was, however, lower than the frequency observed for ciprofloxacin at fourfold the MIC.

To test if targeting gyrase and TopoIV simultaneously could lead to a lower frequency of mutant selection than targeting primarily one enzyme, we compared the frequency of selection of mutants with ciprofloxacin, which preferentially targets TopoIV, with WCK-1734, which preferentially targets gyrase, and with the combination of the two. We measured the frequency of mutants selected with ciprofloxacin and WCK-1734 separately at twofold their respective MICs and the two drugs together, each at its MIC. We hypothesized that if dual targeting of TopoIV and gyrase is an effective mechanism to decrease the frequency of selection of mutants, then the frequency of mutants selected with ciprofloxacin combined with WCK-1734 would be substantially lower than the frequency of selection with each drug alone. We tested the number of mutants selected for each drug or combination of drugs simultaneously from each of six independent cultures of ISP794. The ratio of the frequencies of mutants selected with ciprofloxacin and with WCK-1734 alone at twofold their MICs did not differ significantly from each other (*t* test, $P = 0.172$) (mean, 24.9; median, 4.5; range, 1.5 to 91.8). In contrast, the ratio of frequencies of mutants selected with WCK-1734 alone at twofold its MIC with those selected with the combination (each at its MIC) was significantly greater than unity (*t* test, $P = 0.008$) (mean, 33.7; median, 33.7; range, 9.2 to 57.4). Mutants could not be selected with the combination at more than 1.5-fold the MIC of each of the drugs ($<2 \times 10^{-11}$ to 4×10^{-11}).

Characterization of single-step mutants selected by the combination of ciprofloxacin and WCK-1734. To gain information on how dual targeting affects patterns of resistance mutations, we characterized mutants selected with the combination of ciprofloxacin and WCK-1734. We found no mutations in seven different resistant mutants selected at the MIC of ciprofloxacin and WCK-1734 on sequencing nucleotides 1 to 564 and 1 to 573 of *parC* and *gyrA*, respectively, a region that

TABLE 5. WCK-1734 and ciprofloxacin inhibition of catalytic activity and stimulation of cleavage complex formation with TopoIV and gyrase

Drug	IC_{50} (μg/ml)[a]		CC_{50} (μg/ml)[b]	
	Topo IV	Gyrase	Topo IV	Gyrase
WCK-1734	2.5–5.0	1.25	2.5–5.0	0.62
Ciprofloxacin	1.25–2.5	5.0	1.25–2.5	30–50

[a] IC_{50}, drug concentration causing half-maximal inhibition of decatenation of kDNA minicircle for TopoIV and supercoiling of relaxed pBR322 for gyrase.
[b] CC_{50}, concentration causing half-maximal intensity of linear plasmid pBR322 DNA.

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TABLE 6. Frequency of selection of resistant mutants of strain ISP794

Concentration (μg/ml) of selecting drug (factor of MIC of selecting drug)		Frequency of selection of mutants		
WCK-1734	Ciprofloxacin	WCK-1734	Ciprofloxacin	WCK-1734 plus ciprofloxacin
0.008 (1)		8.9×10^{-7}–8.5×10^{-6}		
0.016 (2)		1.9×10^{-9}–8.0×10^{-7}		
0.024 (3)		7.5×10^{-11}–8.3×10^{-10}		
0.032 (4)		$<3.2 \times 10^{-11}$		
	0.25 (1)		8.0×10^{-6}–3.0×10^{-5}	
	0.5 (2)		2.4×10^{-7}–7.8×10^{-6}	
	1.0 (4)		8.5×10^{-9}–7.7×10^{-8}	
	1.25 (5)		4.6×10^{-10}–5.4×10^{-10}	
	2.0 (8)		$<2.6 \times 10^{-11}$	
0.008 (1)	0.25 (1)			1.1×10^{-9}–4.7×10^{-8}
0.012 (1.5)	0.375 (1.5)			$<3.7 \times 10^{-11}$–2.5×10^{-9}
0.016 (2)	0.5 (2)			$<2.6 \times 10^{-11}$

includes the QRDRs of these two genes. We therefore selected for resistant mutants at 1.5-fold MIC (ciprofloxacin, 0.375 μg/ml; WCK-1734, 0.012 μg/ml), the highest concentration at which mutants could be detected. We further characterized three of these mutants as well as two of the first seven mutants. For strains 1734/cip-A and 1734/cip-Y the MICs of ciprofloxacin and of WCK-1734 increased concordantly twofold or not at all, and strain 1734/cip-Y was the only mutant that manifested a two- to fourfold increase in the MIC of the combination of ciprofloxacin and WCK-1734. Of the remaining three strains, 1734/cip-P had a twofold and 1734/cip-T and 1734/cip-W had threefold increased MICs for each of the combination drugs. Strain 1734/cip-T was exceptional in its eightfold increase in the MIC of ciprofloxacin, while maintaining the wild-type MIC of WCK-1734. The MIC of neither drug decreased in the presence of reserpine. Sequencing the entirety of *parC*, *parE*, *gyrA*, and *gyrB* of the five mutants revealed only two mutations. One was Gly30Arg in *parE* that did not cause a change in the MIC of individual drugs in mutant 1734/cip-P. Interestingly, in mutant 1734/cip-T, which had an increased MIC of ciprofloxacin, we identified a novel Phe18Leu mutation in *parC*, upstream of the QRDR. Although the four strains selected on WCK 1734 alone had *gyrA* mutations, none of the five mutants derived from combination drug selection had a mutation in *gyrA*, suggesting that the presence of ciprofloxacin in the combination may have prevented selection of *gyrA* mutations.

DISCUSSION

We have determined the target specificity of WCK-1734 in *S. aureus* by using established *gyrA* and *parC* mutants, by analysis of single-step mutants selected with WCK-1734, and by measurements of drug activity against both gyrase and TopoIV. A key finding was that single mutations in *gyrA* but not *parC* increased the MIC of WCK-1734, suggesting that gyrase is the primary target for WCK-1734 in *S. aureus*. Analysis of mutants 1734-J and 1734-S selected with WCK-1734 also supports this conclusion. Allelic exchange experiments demonstrated that the GyrA Gly82Asp mutation in these mutants, which were novel in *S. aureus*, was responsible for resistance, findings that are consistent with the resistance phenotype conferred by the homolog of this mutation in *E. coli* (3). Furthermore, in each of four characterized mutants selected with WCK-1734, a mutation in *gyrA* was found, and in no case were *parC* and *parE* mutants alone identified after selection with WCK-1734.

Biochemical studies also showed that WCK-1734 inhibited *S. aureus* gyrase more effectively than TopoIV, with IC_{50}s of 1.25 μg/ml for inhibition of gyrase-mediated negative supercoiling and 2.5 to 5 μg/ml for inhibition of TopoIV-mediated decatenation. Similarly, WCK-1734 was more effective in stimulating cleavage complex formation with gyrase than with TopoIV, with CC_{50}s of 0.62 μg/ml and 2.5 to 5.0 μg/ml, respectively. The assays of inhibition were optimized for each enzyme, and thus comparisons between enzymes were not under identical conditions (8, 10, 29, 35). Comparisons of the relative activity of WCK-1734 and ciprofloxacin for each enzyme assay were comparable, however, and agree with the in vivo results. Ciprofloxacin was more effective than WCK-1734 in inhibiting decatenation by and stimulating cleavage complex formation with TopoIV, and WCK-1734 was more effective than ciprofloxacin in inhibiting negative supercoiling by and stimulating cleavage complex formation with gyrase. Taken together, the biochemical and genetic data strongly support a preference of WCK-1734 for targeting gyrase over TopoIV.

The structure of WCK-1734 differs from that of ciprofloxacin in two moieties: a methyl in position C-8 of the quinoline ring and a 4-hydroxy-3-methyl-1-piperidinyl moiety at C-7. T-3912 is a nonfluorinated quinolone developed for topical use that has a C-8-methyl group like that of WCK-1734. The MIC of T-3912 did not increase for a ParC S83F mutant of *S. aureus*, whereas it increased twofold for a GyrA E88G mutant, suggesting that it, too, has gyrase as its primary target in *S. aureus* (38). Changes in C-8 dramatically also alter the initial target of fluoroquinolones in pneumococci (31). A hydrogen at C-8, as in ciprofloxacin, is associated with high activity against TopoIV. In contrast, a halogen substituent appears to shift the initial target to DNA gyrase and markedly reduces anti-TopoIV activity, as in the case of sparfloxacin (27). Significantly, the topical fluoroquinolone nadifloxacin, which bears a 4-hydroxy-1-piperidinyl moiety (a homolog of the C-7 substituent of WCK 1734) is reported to have gyrase as its primary target in *S. aureus* (35). In addition, gatifloxacin and moxifloxacin,

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both of which have methoxy substituents at C-8, select for *gyrA* first-step mutants in pneumococci (9, 30). Interestingly, however, gatifloxacin and moxifloxacin in *S. aureus* select TopoIV mutants (18, 19). More structure-function studies are needed, however, to elucidate the role of C-8 methyl and C-7 substituents in determining the primacy of enzyme targeting in *S. aureus*.

Having identified the differences in quinolone target preference for WCK-1734 and ciprofloxacin, we then addressed the question of whether dual targeting with a combination of two quinolones with differential target preferences would have an effect on the frequency and types of mutants selected, as predicted by the dual-targeting paradigm. Using two quinolones to provide simultaneous attack of both gyrase and TopoIV, we demonstrated directly that dual targeting of TopoIV and gyrase reduces the frequency of selection of resistant mutants, as has been predicted. By using the same drugs to compare primarily single enzyme attack and dual targeting, we were able to circumvent the possibility in single drug tests (14, 15, 17-19) that other properties of the drug (as manifested by the selection of unusual mutations) in addition to dual targeting could have resulted in a low frequency of mutant selection. Furthermore, the indifferent interactions between WCK-1734 and ciprofloxacin in vivo and in CC formation suggest that unique drug-drug interactions cannot account for the findings.

The frequencies of mutants selected with ciprofloxacin and WCK-1734 separately at twofold their MICs and combined with each other at their respective MICs and the characteristics of the different selected strains are revealing in several respects. First, the activities of ciprofloxacin and WCK-1734 against genetically defined mutants and the mutations found in the single-step, single-drug-selected mutants are consistent with a more differential effect of ciprofloxacin than of WCK-1734 on the two target enzymes. Thus, the order of dual-targeting activities of the drugs would be ciprofloxacin plus WCK-1734 > WCK-1734 > ciprofloxacin. The increasing difficulty in selecting mutants at increasing MICs is in agreement with this target preference order, as well as with previous studies on drugs with dual-targeting properties in which selection of mutants proved difficult at concentrations above the MIC (18, 19, 29). Second, single-step mutants selected with various quinolones manifest an interesting pattern of increases in the MIC relative to the parent strain, ISP794: 32-fold with ciprofloxacin (36); eightfold with moxifloxacin (17), garenoxacin (18), and WCK-1734 (present study); and fourfold with gemifloxacin (19). Similarly, for wild-type *S. pneumoniae*, the MICs of ciprofloxacin and sparfloxacin in first-step mutants increased eightfold (27) but only twofold for clinafloxacin (29), suggesting that the more balanced dual-targeting drugs result in progressively lower increments in the MIC for single-step mutants. Our findings were consistent with this pattern; the MIC of the single-step mutants selected with the combination of ciprofloxacin and WCK-1734 increased only two- to fourfold. Third, this pattern is also in agreement with the concept of steps leading to quinolone resistance by mutations in the two topoisomerase target enzymes. After first mutation in the more sensitive enzyme, the MIC is determined by the sensitivity of the second enzyme. If the sensitivities to the two enzymes are similar, then the MIC is expected to change minimally with a single target mutation (13). These dual-targeting effects result in a lowering of the mutant prevention concentration in relation to the MIC (4).

Additional support for the dual-targeting activity of the combination of WCK-1734 and ciprofloxacin comes from our finding that 8 of the 10 independent, first-step resistant mutants selected with the combination of ciprofloxacin and WCK-1734 lacked any mutation in either the TopoIV or gyrase subunits, in contrast to the findings for the mutants selected with either drug alone. In addition, we did not detect mutations in the promoter regions of *parE* or *gyrB*, the former of which has been recently described as a quinolone resistance mechanism through reduced enzyme expression (16). Of note, these mutants also did not manifest other known mechanisms of resistance for quinolones in that there was no effect of reserpine, an inhibitor of NorA and other efflux pumps, on the MIC of either drug. Similar findings were reported for first-step mutants of *S. aureus* selected with gemifloxacin (19) and garenoxacin (18). Thus, the mechanism of resistance found in these rare mutants will require further study.

In summary, we have demonstrated by direct testing the dual-target paradigm for a reduction in the frequency of selection of resistant mutants with quinolones. We have in addition found evidence to suggest the existence of novel mutational mechanisms for low-level quinolone resistance apart from the alteration of the enzyme targets and the effects of reserpine-inhibitable efflux pumps. Definition of the nature of these additional resistance mechanisms awaits further study.

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