ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2007, p. 2445–2453 Vol. 51, No. 7
0066-4804/07/$08.00+0 doi:10.1128/AAC.00158-07

Dual Targeting of DNA Gyrase and Topoisomerase IV: Target Interactions of Heteroaryl Isothiazolones in *Staphylococcus aureus*▽

Jijun Cheng, Jane A. Thanassi, Christy L. Thoma, Barton J. Bradbury, Milind Deshpande, and Michael J. Pucci*

Achillion Pharmaceuticals, New Haven, CT 06511

Received 2 February 2007/Returned for modification 20 March 2007/Accepted 3 May 2007

Heteroaryl isothiazolones (HITZs) are antibacterial agents that display excellent in vitro activity against *Staphylococcus aureus*. We recently identified a series of these compounds that show potent bactericidal activities against methicillin-resistant *Staphylococcus aureus* (MRSA). We report here the results of in vitro resistance studies that reveal potential underlying mechanisms of action. HITZs selected *gyrA* mutations exclusively in first-step mutants of wild-type *S. aureus*, indicating that in contrast to the case with most quinolones, DNA gyrase is the primary target. The compounds displayed low mutation frequencies (10^{-9} to 10^{-10}) at concentrations close to the MICs and maintained low MICs (≤0.016 μg/ml) against mutants with single mutations in either *gyrA* or *grlA* (*parC*). These data suggested that HITZs possess significant inhibitory activities against target enzymes, DNA gyrase and topoisomerase IV. This dual-target inhibition was supported by low 50% inhibitory concentrations against topoisomerase IV as measured in a decatenation activity assay and against DNA gyrase as measured in a supercoiling activity assay. Good antibacterial activities (≤1 μg/ml) against staphylococcal *gyrA grlA* double mutants, as well as low frequencies (10^{-9} to 10^{-10}) of selection of still higher-level mutants, also suggested that HITZs remained active against mutant enzymes. We further demonstrated that HITZs exhibit good inhibition of both *S. aureus* mutant enzymes and thus continue to possess a novel dual-targeting mode of action against these mutant strains. In stepwise acquisition of mutations, HITZs selected quinolone resistance determining region mutations *gyrA*(*Ser84Leu*), *grlA*(*Ser80Phe*), *grlA*(*Ala116Val*), and *gyrA*(*Glu88Lys*) sequentially, suggesting that the corresponding amino acids are key amino acids involved in the binding of HITZs to topoisomerases. The overall profile of these compounds suggests the potential utility of HITZs in combating infections caused by *S. aureus*, including multidrug-resistant MRSA.

Staphylococcus aureus is a key gram-positive pathogen that can cause life-threatening infections. Effective treatment by antistaphylococcal agents has become increasingly compromised by the emergence of antibiotic-resistant strains, particularly methicillin-resistant *S. aureus* or MRSA (13, 35, 39). MRSA strains are often resistant to multiple antibiotics and pose serious challenges in hospitals and increasingly now in the community (14, 15, 37). One response to the drug resistance problem is to develop new and more effective antibiotics. Although several new agents, such as daptomycin and linezolid, have been approved in recent years, the ongoing specter of antibiotic resistance creates an uncertain future for all current antibacterials (10, 17, 33, 38, 41).

Following introduction of nalidixic acid in 1962, the quinolones have been extensively evaluated and improved as a class of antibacterial agents with broad-spectrum activity (18, 21). They act by forming cytotoxic ternary complexes with bacterial genomic DNA and two essential topoisomerases, DNA gyrase and topoisomerase IV. Resistance studies with a number of quinolones correlated target preference to the first selected mutation, occurring usually in the primary or more-sensitive enzyme target (20, 43). Based on such studies and in vitro activities, most quinolones were found to preferentially target topoisomerase IV in *S. aureus* (18, 19, 23, 49). Several of the more recent quinolones on the market, such as gatifloxacin, moxifloxacin, and gemifloxacin, have been optimized for improved gram-positive antibacterial activity compared with earlier quinolones, such as ciprofloxacin (2–4, 26, 28). However, because of increased resistance to fluoroquinolones, primarily mediated by chromosomal mutations in the genes encoding the target enzymes, none of these drugs can achieve sufficient in vivo exposure to effectively treat most clinical infections caused by MRSA.

Heteroaryl isothiazolones (HITZs), particularly the isothiazoloquinolones, represent a class of compounds with structural similarities to the quinolones. Previous work reported that the HITZs possessed excellent antibacterial activity against key pathogens, including staphylococci (7, 8). We have recently evaluated the antibacterial potencies of several new HITZ derivatives and identified compounds that are highly bactericidal against antibiotic-resistant MRSA strains both in vitro and in animal infection models (34). In this work, we characterized target inhibition and resistance to the HITZs in an effort to further understand the mechanism of action of these highly active compounds. Results indicated that HITZs exclusively select first-step gyrase mutations in *S. aureus* with mutation frequencies that are quite low. This is in contrast to most

* Corresponding author. Mailing address: Achillion Pharmaceuticals, 300 George Street, New Haven, CT 06511. Phone: (203) 624-7000. Fax: (203) 624-7003. E-mail: mpucci@achillion.com.

▽ Published ahead of print on 14 May 2007.

TABLE 1. Strains, genotypes, and sources

Strain	Genotype or description	Source or reference
ATCC 29213	CSCI QC reference strain	ATCC
ACH-0204	29213[a] *gyrA*(*Ser84Leu*)	This study; compound 3-R of ATCC 29213[b]
ACH-0216	29213 *grlA*(*Ser80Phe*)	This study; ciprofloxacin-R of ATCC 29213
ACH-0126	29213 *gyrA*(*Ser84Leu*) *grlA*(*Ser80Phe*)	This study; compound 3-R of ACH-0204
ACH-0192	29213 *gyrA*(*Ser84Leu*) *grlA* (*Ser80Phe*, *Ala116Val*)	This study; compound 4-R[c] of ACH-0126
ACH-0203	29213 *gyrA*(*Ser84Leu*) *grlA*(*Ser80Phe*, *Ala116Val*)	This study; compound 4-R of ACH-0192
ACH-0129	29213 *gyrA*(*Ser84Leu*, *Glu88Val*) *grlA*(*Ser80Phe*, *Ala116Val*)	This study; compound 3-R of ACH-0126
ACH-0130	29213 *gyrA*(*Ser84Leu*, *Glu88Val*) *grlA*(*Ser80Phe*, *Ala116Val*)	This study; compound 3-R of ACH-0129
ACH-0141	29213 *gyrA*(*Ser84Leu*, *Glu88Val*) *grlA*(*Ser80Phe*, *Ala116Val*)	This study; compound 3-R of ACH-0130
ACH-0206	29213 *gyrA*(*Ser84Leu*)	This study; compound 1-R of ATCC 29213
ACH-0210	29213 *gyrA*(*Ser84Leu*) *grlA*(*Ser80Phe*)	This study; compound 1-R of ACH-0206
ACH-0186	29213 *gyrA*(*Ser84Leu*) *grlA*(*Ser80Phe*, *Ala116Val*)	This study; compound 1-R of ACH-0210
ACH-0201	29213 *gyrA*(*Ser84Leu Glu88Val*) *grlA*(*Ser80Phe*, *Ala116Val*)	This study; compound 1-R of ACH-0186
ATCC 33591	*mecA*$^+$	ATCC; GenBank accession no. BA000017
ACH-0218	33591 *grlA*(*Glu88Lys*)	This study; ciprofloxacin-R of ATCC 33591
ACH-0221	33591 *gyrA*(*Ser84Leu*)	This study; compound 1-R of ATCC 33591
NY2746	*mecA*$^+$ *gyrA*(*Ser84Leu*) *grlA*(*Ser80Phe*)	27
ATCC 700699	*mecA*$^+$ *gyrA*(*Ser84Leu*, *Glu409Lys*) *grlA*(*Ser80Phe*)	ATCC
BSA643	*mecA*$^+$ *gyrA*(*Ser84Leu*) *grlA*(*Ser80Tyr*, *Glu84Gly*)	27
BSA678	*mecA*$^+$ *gyrA*(*Ser84Leu*, *Ser85Pro*) *grlA*(*Ser80Phe*, *Glu84Lys*)	27
ACH-0231	*mecA*$^+$ *gyrA*(*Ser84Leu*, *Glu88Lys*) *grlA*(*Ser80Tyr*, *Glu84Gly*)	Focus BioInova, Herndon, VA

[a] "29213" indicates that the strain ACH-0204 was derived from *S. aureus* ATCC 29213.
[b] "Compound 3-R of ATCC 29213" indicates that ACH-0204 is a spontaneously derived mutant of ATCC 29213 selected on solid medium for resistance to compound 3.
[c] For compound 4 structure, see Fig. 1.

quinolones, which select first-step topoisomerase IV mutations in staphylococci. Results also indicated that HITZs select topoisomerase IV mutations in later steps of high-level resistance. This dual targeting of gyrase as well as topoisomerase IV is reflected in good inhibitory activity against these enzymes as measured by in vitro assays. We believe that their potential as antibacterial agents merits further study for this class of compounds.

(This work was presented in part at the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 2006 [6].)

MATERIALS AND METHODS

Media, antibacterial agents, *S. aureus* strains, and culture conditions. *S. aureus* strains were grown in brain heart infusion (BHI), Mueller-Hinton (MH), or Mueller-Hinton II medium obtained from BD Biosciences (San Jose, CA). Ciprofloxacin was purchased from ICN Biomedicals (Costa Mesa, CA). Ethidium bromide was purchased from Invitrogen (Carlsbad, CA). Moxifloxacin was purchased from LKT Laboratories (St. Paul, MN). Gemifloxacin and compounds 1, 2, 3, and 4 were prepared internally at Achillion Pharmaceuticals. *S. aureus* strains used in this work are listed in Table 1.

Growth curves and determination of doubling times. *S. aureus* strains were cultured in MH or Mueller-Hinton II medium at 37°C with aeration. Overnight cultures were diluted to an optical density at 625 nm of approximately 0.05, and turbidity was monitored every 30 min over the first 7 h. Bacterial growth curves (log optical density at 625 nm versus time) were plotted to estimate doubling time (T_D).

Drug susceptibility assays. MICs were determined by a broth microdilution technique according to CLSI (formerly NCCLS) approved guidelines (30). The MIC was defined as the lowest concentration of an antimicrobial agent that prevented visible growth at 24 h. All MICs were determined at least twice.

Resistant mutant selection. Parent strains were cultured in BHI broth overnight and concentrated in a 1/10 volume of phosphate-buffered saline, followed by plating of approximately 1×10^{10} CFU onto BHI agar plates containing a 0.5×, 1×, 2×, 4×, or 8× MIC concentration of HITZs or quinolones, followed by incubation at 37°C for 24 h for relatively normally growing mutants or longer for slow-growing mutants. In every stepwise mutant selection, *S. aureus* cell density was determined by plating serial 10-fold dilutions on BHI agar. Selection frequencies for resistant mutants were calculated as the ratio of the number of resistant colonies to the total cell number. Mutation analyses were done at least twice. Mutant colonies were streaked to purity on BHI agar plates containing the same selecting drug concentrations. Once a first-step resistant mutant was confirmed by determining the MIC, the selection procedure was repeated serially to generate second-step and further-step mutants.

Mutant stability and growth adaptation assessment. To check for stability, in the absence of drug selective pressure, of the third- and fourth-step mutants ACH-0129 and ACH-0130, a single colony of each strain was grown in 5 ml of BHI broth in the absence of drug at 37°C overnight and single colonies were recovered on BHI agar. This procedure was repeated for 20 passages, and colonies were tested for HITZ and quinolone MICs and for culture doubling times. To assess possible growth adaptation of the third-step mutant, ACH-0201, the strain was cultured in 5 ml BHI broth in the absence or presence of compound 1 (1 μg/ml, equal to 0.5× MIC) for 24 h. Ten microliters of this culture then was inoculated into 5 ml fresh BHI broth with or without compound 1 (1 μg/ml) and cultured for 24 h. This process was repeated 20 times, and cultures were streaked on BHI agar plates in the presence or absence, respectively, of compound 1 (1 μg/ml) to obtain single colonies, which were then used to determine MICs and doubling times.

DNA sequencing. Template genomic DNAs corresponding to the *S. aureus* quinolone resistance determining regions (QRDRs) of *gyrA*, *gyrB*, *grlA*, and *grlB* (11, 27) and to the coding and flanking regions of *norA* were amplified and sequenced by standard methods. Sequencing was done by automated ABI 3100 DNA sequencers at the W.M. Keck Facility (Yale University, New Haven, CT). Sequences of oligonucleotides used to amplify and sequence the QRDR regions of *gyrA*, *gyrB*, *grlA*, and *grlB* were as previously described (11). Oligonucleotide pairs (5′ CTCGTCAATTCCAGTGGCTCAG and 5′ CATAAGAAAAACGA TGCTAATCATTCA) were used to amplify and sequence a 1.8-kb genomic DNA fragment of *norA* containing the 400-bp 5′ untranslated region, the entire open reading frame, and the 50-bp 3′ untranslated region and were also used to sequence the *norA* fragment.

Cloning, protein expression, and purification. Genes encoding the wild-type proteins GyrA, GyrB, GrlA, and GrlB and mutant proteins GyrA Ser84Leu and GrlA Ser80Phe were cloned into pET vector overexpression constructs with six-His tags (Novagen, EMD, La Jolla, CA). The GyrA proteins were expressed in a modified pET vector encoding the cleavage site for tobacco etch virus protease downstream of the thioredoxin gene (42). Proteins were expressed in *Escherichia coli* BL21(DE3)(pLysS) cells and purified individually to greater than 95% homogeneity from the supernatant of lysed cells by nickel affinity based on the ProBond protocol from Invitrogen, followed by size exclusion column chro-

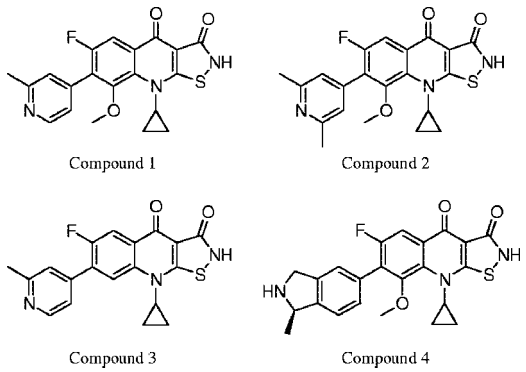

FIG. 1. Structures of heteroaryl isothiazolones used in this study.

matography. The GyrA protein was cleaved with recombinant tobacco etch virus protease (Invitrogen, Carlsbad, CA) during purification.

Topoisomerase IV assay. Wild-type and mutant topoisomerases IV were reconstituted in vitro by incubating GrlA (wild type and Ser80Phe mutant) and GrlB proteins (1:1 molar ratio) on ice for 10 min. Topoisomerase IV activity was measured by using a decatenation assay that monitored the ATP-dependent unlinking of DNA minicircles from kinetoplast DNA (kDNA) (TopoGEN, Inc., Port Orange, FL). Specifically, 0.1 μg of catenated kDNA was incubated with 2 U of *S. aureus* topoisomerase IV for 30 min at 37°C in 20 μl of the following: 1 mM ATP, 5 mM dithiothreitol, 5 mM $MgCl_2$, 50 μg/ml bovine serum albumin (BSA), 50 mM Tris-HCl (pH 7.5), and 250 mM potassium glutamate. A unit of enzyme is defined as the smallest amount of protein that decatenates 50% of 0.1 μg kDNA in a linear time range over 60 min. Reactions were stopped with 2 μl 0.5 M EDTA and 3 μl DNA loading buffer, and the total reaction was separated on a 1% agarose/Tris-borate-EDTA (TBE) gel for 16 h at 25 V. Gels were stained with 0.5 μg/ml ethidium bromide in TBE buffer for 45 min and destained with water for 1 h. DNA was quantitated with an Alpha Imager 2200 analysis system. The 50% inhibitory concentrations (IC_{50}s) of HITZs and quinolones were determined by nonlinear regression analysis with Graphpad Prism software (Graphpad Software, Inc., San Diego, CA).

DNA gyrase assay. Wild-type and mutant DNA gyrases were reconstituted in vitro by incubating GyrA (wild type or Ser84Leu mutant) and GyrB proteins (slightly below a 1:1 ratio) on ice for 10 min. Gyrase activity was measured by a supercoiling assay that monitored the ATP-dependent conversion of relaxed pBR322 DNA to the supercoiled form. Specifically, 0.1 μg of relaxed pBR322 DNA (TopoGEN) was incubated with 1 U of *S. aureus* DNA gyrase for 60 min at 37°C in 20 μl of the following: 2 mM ATP, 7.5 mM dithiothreitol, 30 mM KCl, 7.5 mM $MgCl_2$, 75 μg/ml of BSA, 75 mM Tris-HCl (pH 7.5), and 300 mM potassium glutamate. A unit of enzyme is defined as the smallest amount of protein that supercoils 50% of 0.1 μg relaxed pBR322 DNA in a linear time range. Reactions were stopped with 10 μl 0.5% sodium dodecyl sulfate–6 mM EDTA–5.35% glycerol–0.013% bromophenol blue, and the total reaction product was loaded onto a 1% agarose-TBE gel. Gel electrophoresis, staining, destaining, image capture, and analysis were done as described above.

RESULTS

Activities of heteroaryl isothiazolones against laboratory-derived mutants. Structures of HITZs and quinolones used in this study are shown in Fig. 1. To study the targets for these compounds in bacteria, we first assessed their activities against a panel of laboratory mutant strains of *S. aureus* derived by single selection on solid medium with characterized lesions in the QRDRs of reported quinolone target genes: DNA gyrase, subunits *gyrA* and *gyrB*, and topoisomerase IV, subunits *grlA* and *grlB* (20, 24, 44). The *grlA*(*Ser80Phe*) single-mutant strain, ACH-0216, was eight- and fourfold more resistant to ciprofloxacin and moxifloxacin, respectively, than the wild type, while the *gyrA*(*Ser84Leu*) mutant strain, ACH-0204, was only twofold less susceptible to these comparator quinolones (Table 2). This is consistent with previous reports that ciprofloxacin and moxifloxacin primarily target topoisomerase IV in *S. aureus* (23). The MICs of gemifloxacin were fourfold higher against each of the strains carrying single mutations, consistent with a previous report that gemifloxacin dually targets both gyrase and topoisomerase IV in *S. aureus* (24). In contrast, the MICs of compounds 1,2, and 3 against the two single-mutant strains were elevated two- and fourfold, respectively, relative

TABLE 2. Characteristics of laboratory-derived mutants of *S. aureus* ATCC 29213 and resistant *S. aureus* clinical isolates

Strain	MIC (μg/ml)[a]							T_D[c] (min)	Mutation(s) in QRDR encoded by[b]:	
	Compound 1	Compound 2	Compound 3	CIP	MOX	GEM	EB		*gyrA*	*grlA*
ATCC 29213	0.004	0.004	0.008	0.25	0.06	0.03	4	30		
ACH-0204	0.016	0.016	0.03	0.5	0.125	0.125	4	30	Ser84Leu	
ACH-0206	0.015	0.015	0.03	0.5	0.125	0.125	4	30	Ser84Leu	
ACH-0221	0.03	0.03	0.03	1	0.5	0.5	16	ND	Ser84Leu	
ACH-0216	0.008	0.008	0.016	2	0.25	0.125	4	30		Ser80Phe
ACH-0218	0.008	0.004	0.08	2	0.25	0.125	16	ND		Glu84Lys
ACH-0126	0.125	0.125	0.5	64	4	4	4	34	Ser84Leu	Ser80Phe
ACH-0210	0.125	0.125	0.5	64	4	4	2	30	Ser84Leu	Ser80Phe
ACH-0186	0.5	0.25	0.5	64	16	4	1	34	Ser84Leu	Ser80Phe, Ala116Val
ACH-0192	0.5	1	2	64	16	8	2	121	Ser84Leu	Ser80Phe, Ala116Val
ACH-0203	1	1	2	128	32	32	32	338	Ser84Leu	Ser80Phe, Ala116Val
ACH-0129	0.5	0.5	4	64	8	8	4	40	Ser84Leu, Glu88Val	Ser80Phe, Ala116Val
ACH-0130	1	1	8	256	32	32	32	52	Ser84Leu, Glu88Val	Ser80Phe, Ala116Val
ACH-0201	2	1	8	64	16	8	2	180	Ser84Leu, Glu88Val	Ser80Phe, Ala116Val
ACH-0141	2	2	16	64	16	4	4	340	Ser84Leu, Glu88Val	Ser80Phe, Ala116Val
ATCC 33591	0.004	0.004	0.004	0.5	0.125	0.06	16	ND		
NY2746	0.25	0.25	0.5	256	8	32	16	33	Ser84Leu	Ser80Phe
ATCC 700699	0.25	0.125	0.25	64	4	4	>64	39	Ser84Leu, Glu409Lys	Ser80Phe
BSA643	0.125	0.125	0.25	256	16	32	16	38	Ser84Leu	Ser80Tyr, Glu84Gly
BSA678	0.5	0.5	4	128	>64	64	4	42	Ser84Leu, Ser85Pro	Ser80Phe, Glu84Lys
ACH-0231	2	1	16	>64	32	>64	16	35	Ser84Leu, Glu88Lys	Ser80Tyr, Glu84Gly

[a] CIP, ciprofloxacin; GEM, gemifloxacin; MOX, moxifloxacin; EB, ethidium bromide.
[b] No mutations found in *gyrB* or *grlB* (*parE*).
[c] T_D, doubling time of bacterial growth.

to those for the wild type, suggesting that these compounds might also effectively target both enzymes in S. *aureus* but with a possible preference for DNA gyrase.

We next examined the activities of HITZs against *gyrA grlA* double mutants, using laboratory strain ACH-0126. The *gyrA*(*Ser84Leu*) *grlA*(*Ser80Phe*) *gyrA grlA* double mutant was consistently selected as a second-step mutant in our study (Table 2) and also by quinolones as described in numerous previous reports (19, 36, 40). Such double mutations are also prevalent in quinolone-resistant MRSA clinical isolates (19, 22). Compounds 1, 2, and 3 displayed MICs of 0.125, 0.125, and 0.5 μg/ml, respectively, against ACH-0216, while the MICs of ciprofloxacin, moxifloxacin, and gemifloxacin against this double mutant were much higher: 64, 4, and 4 μg/ml, respectively. Therefore, all three HITZs remained quite active compared to the quinolones. The MICs of the HITZs against ACH-0126 were 32- to 64-fold higher than those against the wild-type strain, whereas MICs of quinolones increased 64- to 256-fold. This suggested that the HITZs might be better inhibitors of mutant gyrase and/or mutant topoisomerase IV in strains such as ACH-0126.

Using this double mutant for further stepwise selection by HITZs, triple (ACH-0192 and ACH-0203) and quadruple (ACH-129, ACH-0130, and ACH-0141) mutants were obtained. The third mutation, *grlA*(*Ala116Val*), detected in ACH-0192 was associated with four- to eightfold MIC increases for the HITZs compared with MICs against the double mutant ACH-0126. This same mutation also resulted in four- and twofold MIC elevations of moxifloxacin and gemifloxacin, respectively, yet the MIC of ciprofloxacin did not increase. The *grlA*(*Ala116Val*) enzyme exhibited cross-resistance to HITZs and more recent quinolones, including moxifloxacin and gemifloxacin, but not to the earlier quinolone ciprofloxacin, suggesting that *grlA*(*Ala116*) may be required in addition to *grlA*(*Ser80*) for more efficient binding of HITZs, moxifloxacin, and gemifloxacin but not ciprofloxacin to topoisomerase IV. A fourth mutation, *gyrA*(*Glu88Val*), found in strain ACH-0129, increased the MIC of compound 3 only by an additional twofold, whereas the MICs of the other two HITZs or the three quinolones tested did not increase compared with those for strain ACH-0192, which contains only three mutations (Table 2).

These multistep mutants were sometimes associated with additional resistance to ethidium bromide, an indication of possible gain-of-function *norA* efflux mutations (Table 2). However, DNA sequence analyses of both the promoter region and the full-length *norA* gene of both strains ACH-0203 and ACH-0130 detected none of the reported *norA* mutations causing resistance to ethidium bromide and ciprofloxacin (31, 32). There remains the possibility that other efflux mutations are located elsewhere and result in overexpression of NorA, as previously reported (45–47). Interestingly, the increase in the ethidium bromide MIC observed for the triple mutant ACH-0203 and quadruple mutant ACH-0130 did not change or increased the MICs of HITZs by only twofold relative to those for their wild-type comparator strains. In contrast, mutant strains ACH-0203 and ACH-0130 displayed fourfold-elevated resistance to quinolones. We did not find any additional mutations in the full-length *gyrA*, *gyrB*, *grlA*, *grlB*, or *norA* gene, including promoter regions (data not shown). However, the T_D value of ACH-0141 is more than 4 h, and it is possible that the increase of resistance to HITZs is a consequence of slower growth in this strain.

Activities of heteroaryl isothiazolones against MRSA clinical isolates. To further evaluate HITZs as potential agents to treat quinolone-resistant MRSA, we next determined the MICs of HITZs and comparator quinolones against a panel of clinical MRSA strains with two to four mutations in the QRDRs of *gyrA* and *grlA* and none in the QRDRs of *gyrB* and *grlB* (Table 2). High-level resistance to ethidium bromide was observed for all strains, except for BSA678. In general, they were highly resistant to quinolones, since the lowest MICs were 4 μg/ml for moxifloxacin and gemifloxacin against ATCC 700699. In sharp contrast, HITZs, especially compounds 1 and 2, still possessed good antibacterial activities against all strains, including the two strains with four QRDR mutations (BSA678 and ACH-0231). Even the least-active compound, compound 3, was still active against strains with two or three mutations. Consistent with data in Table 2, the increased ethidium bromide resistance seen in the double mutants NY2746 and ATCC 700699 does not appear to be associated with decreased susceptibility to HITZs, and MICs against the triple mutant BSA643, associated with resistance to ethidium bromide, were not elevated relative to those against the double mutant NY2746.

The triple-mutant strain BSA643 has an additional mutation, *grlA*(*Glu84Gly*), in comparison to double mutants but was still as susceptible to HITZs as the two double mutants NY2746 and ATCC 700699, indicating that the mutation *grlA*(*Glu84Gly*) did not play a major role in resistance. However, the MICs of the three quinolones against BSA643 increased fourfold. The quadruple mutant BSA678 had a fourth additional mutation, *gyrA*(*Ser85Pro*), and displayed wild-type levels of resistance towards ethidium bromide. The MIC of moxifloxacin against this strain increased more than 4-fold compared with that against BSA643, and MICs of HITZs against BSA678 increased 4-fold (16-fold for compound 3) over their MICs against BSA643. Since BSA678 is not associated with resistance to ethidium bromide, this suggests that the *gyrA*(*Ser85Pro*) mutation likely confers at least fourfold resistance to HITZs. Nevertheless, compounds 1 and 2 still retain relatively good antibacterial activity against BSA678, since their MICs were 0.5 μg/ml. Finally, ACH-0231 is a quadruple laboratory mutant with the fourth mutation at *gyrA*(*Ser88Lys*) and is also resistant to ethidium bromide. The MICs of moxifloxacin and gemifloxacin against ACH-0231 increased at least twofold in comparison to their MICs against BSA643, which possesses the identical other three mutations in the QRDRs of *gyrA* and *grlA*. The MICs of compounds 1 and 2 increased 8- to 16-fold against this strain relative to MICs against BSA643. Thus, the *gyrA*(*Glu88Lys*) mutation is likely involved in increased resistance to both HITZs and quinolones. Nevertheless, compounds 1 and 2 still possess antibacterial activity against ACH-0231, with MICs of 1 to 2 μg/ml.

Frequency of first-step mutation selection by compound 1. In vitro resistance studies with compound 1 to determine mutation frequencies were done using a number of staphylococcal laboratory strains, including the wild-type ATCC 29213 and the *gyrA grlA* double-mutant strain, ACH-0126. MRSA strains were also used, including a quinolone-sensitive strain, ATCC

TABLE 3. Frequency of selection of resistant mutants for *S. aureus* laboratory and clinical strains by compound 1

Parent strain	Drug	Selecting drug conc, fold MIC (μg/ml)[a]	Frequency of mutant selection
ATCC 29213	Compound 1	1 (0.004)	1.5×10^{-9}–1×10^{-10}
	Ciprofloxacin	4 (1)	9×10^{-8}–1.2×10^{-8}
	Moxifloxacin	4 (0.25)	6×10^{-8}–2.5×10^{-8}
	Gemifloxacin	2 (0.06)	$<1 \times 10^{-10}$
ATCC 33591	Compound 1	1 (0.004)	5×10^{-9}–2.5×10^{-9}
	Ciprofloxacin	4 (1.0)	3.3×10^{-7}–9.5×10^{-8}
ACH-0126	Compound 1	2 (0.25)	4.4×10^{-10}–$<1.0 \times 10^{-10}$
ATCC 700699	Compound 1	2 (0.5)	$<1 \times 10^{-10}$

[a] Drug concentrations (highest drug concentrations that yielded mutant colonies) are listed both as factors of the MICs and in μg/ml.

TABLE 4. Frequencies of stepwise resistant mutants of *S. aureus* ATCC 29213 selected by compound 1

Step	Strain	Selecting drug conc, fold MIC (μg/ml)[a]	Frequency of mutant selection
Parent	ATCC 29213		
1	ACH-0206	1 (0.004)	1.5×10^{-9}–1.0×10^{-10}
2	ACH-0210	4 (0.064)	9.6×10^{-9}–6.0×10^{-9}
3	ACH-0186	2 (0.25)	4.4×10^{-10}–$>1.0 \times 10^{-10}$
4	ACH-0201	2 (1.0)	1.3×10^{-8}–2.1×10^{-9}

[a] Drug concentrations (highest concentrations that yielded mutant colonies) are listed both as factors of the MICs and in μg/ml.

33591, and a multidrug-resistant strain, ATCC 700699, with *gyrA grlA* mutations. As the MIC ratios of HITZs against *gyrA* or *grlA* single mutants were only twofold compared with those against the wild type (Table 2), we hypothesized that HITZs could effectively target both gyrase and topoisomerase IV. Thus, we would expect low frequencies of mutation selection for wild-type strains by HITZs. We found that the frequency of mutation selection for ATCC 29213, which is in the range of 1×10^{-10} to 1.5×10^{-9} (Table 3), was lower than that by ciprofloxacin, 1.2×10^{-8} to 9×10^{-8}, which was consistent with previously reported mutation frequencies (23). Similarly, the ATCC 33591 mutation selection frequency using compound 1 was 5×10^{-9} to 2.5×10^{-9}, again lower than that, 3.3×10^{-7} to 9.5×10^{-8}, with ciprofloxacin. In addition, compound 1 selected mutants only at concentrations close to 1× MIC, whereas ciprofloxacin and moxifloxacin selected at concentrations of 4× MIC. Consistently, the mutation prevention concentrations (MPCs), defined as the MICs of the least-susceptible single-step mutants (50), of compound 1 against both strains were only two to four times the MIC, compared with eight times the MIC for ciprofloxacin and moxifloxacin (50) (data not shown). Thus, these data are again consistent with a dual-targeting mechanism of action. Similar mutation selection frequency results were seen with other HITZs (data not shown), indicating that other members of the HITZ class share this mechanism of action. Low frequencies of mutation selection and low MPCs for HITZs against ATCC 33591 indicated that the MRSA background did not affect these data.

Interestingly, the frequencies of mutation selection by compound 1 using a laboratory *gyrA grlA* double mutant, ACH-0126, and a clinical MRSA *gyrA grlA* double mutant, ATCC 700699, were both even lower, ranging from 4.4×10^{-10} to $<1 \times 10^{-10}$. Results of selection by compound 2 were essentially in the same low range (data not shown). Since the MICs of ciprofloxacin against both strains are 64 μg/ml, resistance studies of these two strains using ciprofloxacin as a comparator were not performed. Low frequencies of mutation selection together with low MICs would predict that preexisting mutant strains of clinical isolates would not readily acquire additional mutations and become more resistant to these compounds.

Characterization of single-step mutants selected by compound 1. The genotypes of step mutants selected by compound 1 were compared to genotypes of those selected by ciprofloxacin (Table 2). The comparator quinolone ciprofloxacin selected the *grlA*(*Ser80Phe*) mutation (represented by ACH-0216) or *grlA*(*Ser80Tyr*) in multiple mutants of ATCC 29213. It also selected *grlA* mutations, *grlA*(*Glu88Lys*) (represented by ACH-0218), *grlA*(*Ser80Phe*), or *grlA*(*Ala116Pro*), in different isolates of ATCC 33591. All of these mutations were previously reported to be frequently selected by ciprofloxacin (31, 44), and thus, our data were again consistent with reports that ciprofloxacin primarily targets topoisomerase IV in *S. aureus*. In contrast, mutations of *gyrA*(*Ser84Leu*), were detected in multiple first-step mutants of ATCC 29213 (represented by ACH-0206) and ATCC 33591 (represented by ACH-0221) selected by compound 1. In addition, other HITZs exclusively selected the same mutation in both parent strains (data not shown), consistent with the hypothesis that HITZs preferentially target gyrase in *S. aureus*. Since ATCC 33591 is an MRSA strain that displays increased ethidium bromide resistance, our data also suggest that neither methicillin nor ethidium bromide resistance appears to affect selection of gyrase mutations by HITZs in *S. aureus*.

The MICs for the three HITZs were determined against the above mutants and the parent strains and compared with the MICs of several quinolones (Table 2). Compared with the wild type, ATCC 29213, and the *grlA*(*Ser80Phe*) mutant, ACH-0216, the MICs of the HITZs against the *gyrA* mutant were fourfold higher, while those against the *grlA* mutant were twofold higher. Thus, consistent with results in Table 2 and the low frequency of mutant selection shown in Table 3, these data support the contention that despite preferentially targeting gyrase, HITZs possess significant activity against topoisomerase IV. As expected, ratios of MICs for each HITZ against the *gyrA* mutant and the *grlA* mutant of MRSA strain ATCC 33591 were also 2, further illustrating that an MRSA background does not affect activities of HITZs against its targets, gyrase and topoisomerase IV, in *S. aureus*.

Frequencies of selecting additional stepwise mutants of ATCC 29213 by compound 1. The first step mutant, ACH-0206, of ATCC 29213 was selected by compound 1 at a concentration of 1× MIC with a frequency of 1.5×10^{-9} to 1.0×10^{-10} (Tables 3 and 4). Second-step mutants were selected at 0.064 μg/ml, equal to fourfold the MIC of compound 1, using ACH-0206 as the parent strain, with a frequency of 9.6×10^{-9} to 6×10^{-9}. A representative second-step mutant, ACH-0210, was subjected to further mutant selection by compound 1 at a concentration of 0.25 μg/ml (2× MIC) to generate third-step mutants at a frequency of 4.0×10^{-10} to $<1 \times 10^{-10}$. Finally, a representative third-step mutant, ACH-0186, was subjected to additional mutant selection by compound 1 at 1 μg/ml (2× MIC) to generate the fourth-step mutants with a frequency of

1.3×10^{-8} to 2.1×10^{-9}. All of these mutation frequencies were quite low, indicating the difficulty of stepwise acquisition of resistance by selection with compound 1. Similar data for frequencies of stepwise mutant selection were obtained for other HITZs (data not shown), consistent with shared resistance mechanisms. Emergence of fourth-step mutants of compound 1 and other HITZs took more than 3 days and grew poorly (data not shown), raising the possibility that higher-level resistant mutants may not be readily selected in vivo.

Characterization of additional stepwise mutants selected by compound 1. The genotypes of stepwise mutants selected by compound 1 (Table 4) were determined, along with the MICs of HITZs and comparator quinolones against these mutants (Table 2). Similar to other HITZs, compound 1 selected the *gyrA*(*Ser84Leu*) mutation in the first-step mutant and an additional mutation, *grlA*(*Ser80Phe*), in the second-step mutant. Not only was the genotype of the *gyrA*(*Ser84Leu*) mutant ACH-0206 the same as that of ACH-0204 selected by compound 3 (Tables 1 and 2), but also the MICs of HITZs and quinolones against the two strains were identical (Table 2). The same holds true for the *gyrA grlA* double mutant ACH-0210, selected by compound 1, and the *gyrA grlA* double mutant ACH-0126, selected by compound 3 (Table 2). The third-step mutant ACH-0186 had an additional mutation, *Ala116Val*, in *grlA*. It has the same genotype as that of the triple mutant ACH-0192 selected by compound 4 (Table 2). It was found that other HITZs also exclusively selected the third mutation, *grlA*(*A116V*). As expected, the MICs of HITZs and quinolones against ACH-0186 were also the same as those against ACH-0192 (Table 2), indicating that *grlA*(*A116V*) is involved in resistance to HITZs. Finally, the fourth-step mutant, ACH-0201, contained a fourth mutation, *gyrA*(*Glu88Val*). The MIC of compound 1 against this quadruple mutant was 2 μg/ml, the same as that of compound 1 against the quadruple mutant ACH-0141 selected by compound 3 (Table 2). The MICs of quinolones against ACH-0201 were also very similar to those against ACH-0141. In addition, both ACH-0141 and ACH-0201 grew very slowly, with doubling times of 340 and 180 min, respectively. These data suggest that both slow growth and the *gyrA*(*Glu88Val*) mutation in ACH-0201 could contribute to resistance to HITZs (5, 9).

Slow growth and resistance instability of lab mutants. Although the MICs of compounds 1 and 2 against the quadruple mutants ACH-0141 and ACH-0201 were as high as 1 to 2 μg/ml, much lower growth rates were associated with both strains. Passage of ACH-0141 in liquid medium with subsequent reisolation of single colonies in the absence of compound resulted in resistance levels against HITZs and ciprofloxacin reverting back to that of the previous step after just two passages (data not shown). The previous-step mutant, ACH-0130, was not stable either, since within three liquid passages in the absence of drug pressure followed by reisolation of single colonies after each passage, the resistance level dropped back to that of ACH-0129 (data not shown). The same was true for ACH-0201, since after 20 overnight passages in liquid medium in the absence of drug pressure, the level of resistance to HITZs also decreased and growth was improved from a doubling time of 180 min to 74 min (data not shown). Thus, although slow growth was associated with an increase in resistance, the resistance appeared unstable upon removal of selective drug pressure.

TABLE 5. Inhibition of gyrase and topoisomerase IV catalytic activity by HITZs and quinolones

Drug	IC_{50} (μM) of drug for:			
	Gyrase[a]		Topo IV[b]	
	WT[c]	*gyrA* S84L	WT	*grlA* S80F
Compound 2	3.2	21.5	0.8	9.3
Compound 1	1.6	52.3	1.0	15.8
Ciprofloxacin	61.7	>300	3.0	>40
Gemifloxacin	5.6	>300	0.4	9.8
Moxifloxacin	27.5	227	1.0	11.9

[a] Gyrase supercoiling activity.
[b] Topoisomerase IV decatenation activity.
[c] WT, wild-type enzyme.

Activities of HITZs against purified gyrase and topoisomerase IV of *S. aureus*. Based on resistance data, we hypothesized that the potent activities of HITZs against *S. aureus* likely resulted from potent inhibition of gyrase and effective dual targeting of both gyrase and topoisomerase IV. To further investigate this hypothesis, we isolated and purified these enzymes and directly assayed enzyme inhibition by HITZs, as represented by IC_{50}s against the in vitro supercoiling activity of gyrase and the decatenation activity of topoisomerase IV. As shown in Table 5, the IC_{50}s of control quinolones ciprofloxacin, moxifloxacin, and gemifloxacin versus topoisomerase IV in the decatenation assay were 3.0, 1.0, and 0.4 μM, respectively. The low IC_{50}s were consistent with previous reports that quinolones have high binding affinities and potently inhibit staphylococcal topoisomerase IV (23, 24). The IC_{50}s of compounds 1 and 2 were determined to be 1.0 and 0.8 μM, respectively, and were comparable to inhibition levels seen with moxifloxacin (0.8 μM) and gemifloxacin (0.4 μM).

The IC_{50}s of HITZs were also determined versus staphylococcal DNA gyrase in the supercoiling assay. As shown in Table 5, we generated IC_{50}s for the comparator quinolones ciprofloxacin, moxifloxacin, and gemifloxacin of 61.7, 27.5, and 5.6 μM, respectively. The IC_{50}s for compounds 1 and 2 were 1.6 and 3.2 μM, respectively, in a similar range to or slightly more potent than that seen with gemifloxacin. Since gemifloxacin has been reported to be dual targeting and to have the highest activity against gyrase among all commercial quinolones (24), our results support the idea that improved binding affinities for DNA gyrase can significantly contribute to and enhance antibacterial activity against *S. aureus*. This was evident in both supercoiling and "cleavable complex" assays (1) (data not shown). Thus, the enzymatic data are consistent with the resistance data for the HITZs and provide direct evidence that despite preferentially targeting gyrase, HITZs are effective inhibitors of both gyrase and topoisomerase IV at levels that cause severe consequences for the susceptible bacterial cell.

HITZs not only possessed potent activities against wild-type strains but also maintained good activities against *S. aureus gyrA grlA* double-mutant strains (Table 2), suggesting that HITZs still significantly inhibit mutant topoisomerase IV and/or mutant gyrase. To further investigate this possibility, IC_{50}s were determined for HITZs against purified mutant

staphylococcal enzymes. We found that while ciprofloxacin became essentially inactive against mutant topoisomerase IV ($IC_{50} > 40$ μM), the IC_{50}s of the two HITZs, compounds 1 and 2, were 15.8 and 9.3 μM, respectively, similar to those of moxifloxacin and gemifloxacin, 11.9 and 9.8 μM, respectively. Since both moxifloxacin and gemifloxacin target topoisomerase IV and maintained some activities against the *gyrA grlA* mutant (MIC = 4 μg/ml), our data suggested that the HITZs tested can still bind to and at least partially inhibit mutant topoisomerase IV. The activities of HITZs against mutant gyrase were also determined. Interestingly, whereas the three quinolones displayed little or no activity, the IC_{50}s of the HITZs against mutant gyrase were comparable to that of moxifloxacin (52.3 and 21.5 versus 27.5 μM) against the wild-type gyrase. Consistently, the MICs of compounds 1 and 2 against the double mutants, 0.125 μg/ml, were only two to fourfold higher than those of moxifloxacin or gemifloxacin against the wild-type strain, 0.06 and 0.03 μg/ml, respectively. Taking these data together, the more potent antibacterial activities of compounds 1 and 2 likely resulted from an effective dual inhibition of both mutant topoisomerase IV and mutant gyrase, with a more significant effect from inhibition of the latter enzyme.

DISCUSSION

We recently identified HITZ compounds (7) with potent activities against quinolone-resistant MRSA strains (34). Because of structural similarities with the quinolone class, activities against the bacterial targets of quinolones were examined. Mutations in these targets, DNA gyrase and topoisomerase IV, have been shown to lead to resistance against the antibacterial activity of quinolones (23–25). Reduced activity against these target enzymes has been demonstrated in biochemical assays, and the first mutations observed usually correlate with the more-sensitive target (20). Most quinolones primarily target topoisomerase IV in *S. aureus* and usually select first-step mutations in the *grlA* gene, which encodes a subunit of topoisomerase IV. Stepwise acquisition of mutations in *gyrA* and *grlA* progressively reduces the activities of quinolones against their targets (23, 24) and leads to increasing levels of resistance. Such in vitro resistance studies during early clinical development potentially can provide useful information to assist in the prediction of resistance emergence during future clinical use. In an attempt to gain some insights into the mechanisms of antibacterial activity and resistance for *S. aureus*, we studied in vitro resistance development as well as direct measurement of inhibitory activities against purified topoisomerases of *S. aureus.*

HITZs, including compounds 1, 2, and 3, exclusively selected the mutation *gyrA*(*Ser84Leu*) in wild-type *S. aureus.* Yet further resistance studies also indicated that HITZs continued to maintain good activity against topoisomerase IV, as indicated by low selection frequencies and low MPCs and ultimately by the recovery of *grlA* second-step mutations. To further support the hypothesis that HITZs are effective inhibitors of both DNA gyrase and topoisomerase IV, we demonstrated in vitro inhibition of the purified enzymes. IC_{50}s, especially for compounds 1 and 2, against topoisomerase IV decatenation activity were much lower than that of ciprofloxacin and comparable to those of the potent topoisomerase inhibitors moxifloxacin and gemifloxacin (Table 5) (23, 24). Activities of HITZs against gyrase were generally lower by two- to fourfold than that for gemifloxacin, which has been reported to be the most potent gyrase inhibitor among the comparator marketed quinolones (24). Together these data support the potent inhibition of DNA gyrase along with inhibition of topoisomerase IV.

To further understand how HITZs might interact with topoisomerase targets, staphylococcal step mutants were selected and characterized. After the initial gyrase mutation, the second mutations acquired by selection with HITZs were predominantly *grlA*(*Ser80Phe*). Whereas even the most potent quinolones, moxifloxacin and gemifloxacin, lost significant antibacterial activities against this *gyrA grlA* double mutant, HITZs remained very active, since MICs of compounds 1 and 2 against the double mutant were still equal to or lower than that of ciprofloxacin (0.25 μg/ml) against the wild type (Table 2). In addition, frequencies of selecting higher-level mutants of this double mutant by HITZs were extremely low, sometimes $<1.0 \times 10^{-10}$ (Table 3), probably the result of effectively inhibiting two targets, gyrase and topoisomerase IV, simultaneously. Our in vitro analysis of both mutant enzymes suggested that compounds 1 and 2 maintained good inhibitory activities against both mutated staphylococcal enzymes and thus provided direct evidence for the hypothesis that HITZs significantly inhibit both mutant topoisomerases in *gyrA grlA* double mutants (Table 5). We believe that this is the first report to show effective dual-targeting activities of compounds against both mutant enzymes of a *gyrA grlA* double mutant, which is representative of an increasing number of clinical isolates in recent years.

HITZs selected *grlA*(*Ala116Val*) as a third-step mutation and *gyrA*(*Glu88Val*) as a fourth-step mutation. *grlA*-encoded mutations to Glu or Pro at Ala116 have previously been reported as a result of selection by ciprofloxacin or premafloxacin in first-step mutants and were responsible for resistance (25, 31). *gyrA*-encoded mutations to Lys at Glu88 were reported in first-step mutants selected by sparfloxacin or moxifloxacin (16) and second-step mutants selected by ciprofloxacin (12). *grlA*(*Ala116*) and *gyrA*(*Glu88*) substitutions were also present in MRSA strains, with multiple mutations in topoisomerases (22, 32). However, the third- and fourth-step mutations to Val encoded at both *grlA*(*Ala116*) and *gyrA*(*Glu88*) appear to be unique to HITZs and may represent a difference in how HITZs bind to topoisomerases in comparison to quinolones. Nevertheless, the quadruple mutant ACH-0129 was still susceptible to HITZs, such as compounds 1 and 2, since their MICs were both 0.5 μg/ml (Table 2). Although the MICs of compounds 1 and 2 against the quadruple mutants ACH-0141 and ACH-0201 were as high as 1 to 2 μg/ml, severely reduced growth rates were associated with both strains. In the absence of drug pressure, both mutants reverted and lost significant resistance. Thus, although slow growth might have contributed to increased resistance, this resistance appears to be unstable. An additional example is the MRSA clinical isolate BSA678, which also possesses four mutations in the two target genes but has a relatively normal growth rate. This strain still remains susceptible (MIC = 0.5 μg/ml) to compounds 1 and 2 (Table 2). Thus, HITZs in this study appeared to still demonstrate activity against mutant gyrase and/or mutant topoisomerase IV in staphylococcal quadruple mutants.

Mutations resulting in overexpression of *norA*, encoding a major efflux transporter in *S. aureus*, are known to cause significant resistance to hydrophilic quinolones and lipophilic monocationic compounds, such as ethidium bromide (21, 31, 47, 48). Through our screening and evaluations of HITZs, we found that compounds 1, 2, and 3 rarely selected mutants associated with resistance to ethidium bromide. Compounds 3 and 4 selected ethidium bromide resistance only in fourth-step mutants, such as ACH-0203 and ACH-0130. Even so, both strains increased their MICs only by approximately twofold (Table 3). In addition, the MICs of compound 1 or 2 against some clinical MRSA strains expressing resistance to ethidium bromide and with two to four mutations in topoisomerases were found to remain within a twofold range, around 0.25 μg/ml. Therefore, it is possible that HITZs may not be good substrates for at least some efflux transporters in *S. aureus*. This was previously suggested by the observation that MICs remain unchanged with or without the addition of 20 μg/ml reserpine, a known efflux pump inhibitor (34).

About 60% of *S. aureus* infections in U.S. hospital settings are now due to MRSA and are often associated with resistance to other drugs, such as quinolones (29, 34). MRSA strains with at least two topoisomerase mutations have become widespread. For example, NY2746 and ATCC 700699, used in this work, are representative of such MRSA clinical strains that contain mutations *gyrA*(*Ser84Leu*) and *grlA*(*Ser80Phe*) and have become more resistant to quinolones (Table 2). However, HITZs, especially compounds 1 and 2, maintained antibacterial activity against both MRSA strains, with MICs of 0.125 to 0.25 μg/ml, respectively, and also remained active against MRSA strains with three to four mutations in their topoisomerases. Similar to MSSA laboratory strains, the frequency of selection of mutants of MRSA strain ATCC 33591 was low, and it was especially difficult to select higher-level mutants using staphylococcal clinical double mutants, such as MRSA ATCC 700699 (frequency, $<1 \times 1^{-10}$), as parental strains (Table 3). This was in the same range seen with the laboratory double mutant ACH-0126 (frequency = 4.4×10^{-10} to 1×10^{-10}) as the parental strain. The low frequencies of selection of stepwise mutants (Table 4), slow growth, and resistance instability of laboratory mutants with multiple mutations suggest that it may be difficult for HITZs to easily select high-level resistance upon future clinical use.

In summary, HITZ compounds were found to be potent inhibitors of DNA gyrase in staphylococci. Excellent bactericidal activities against wild-type *S. aureus*, low ratios of MICs against *gyrA* or *grlA* single mutants versus wild-type strains, and low frequencies of mutant selection at low drug concentrations together suggest that HITZs effectively target both topoisomerase IV and gyrase in wild-type *S. aureus*. Together with pharmacokinetic profiles that are similar to that of ciprofloxacin in mice (34), we believe that these compounds have the potential to be effective antistaphylococcal agents. Work is in progress to further investigate the heteroaryl isothiazolones as therapeutic options for the treatment of staphylococcal infections.

ACKNOWLEDGMENTS

We acknowledge Akihiro Hashimoto, Edlaine Lucien, Yongsheng Song, Qiuping Wang, and Jason Wiles for synthetic chemistry efforts. We also thank Donald Low and Darrin Bast for supplying MRSA clinical isolates. We thank Yangsi Ou for technical assistance and Steve Podos for helpful discussions.

REFERENCES

1. **Barrett, J. F., J. I. Bernstein, H. M. Krause, J. J. Hilliard, and K. A. Ohemeng.** 1993. Testing potential gyrase inhibitors of bacterial gyrase: a comparison of the supercoling inhibition assay and "cleavable complex" assay. Anal. Biochem. **214:**313–317.
2. **Bauernfeind, A.** 1997. Comparison of the antibacterial activities of the quinolones Bay 12-8039, gatifloxacin (AM 1155), trovafloxacin, clinafloxacin, levofloxacin and ciprofloxacin. J. Antimicrob. Chemother. **40:**639–651.
3. **Blondeau, J. M.** 2000. Gatifloxacin: a new fluoroquinolone. Expert Opin. Investig. Drugs **9:**1877–1895.
4. **Blondeau, J. M., R. Laskowski, J. Bjarnason, and C. Stewart.** 2000. Comparative in vitro activity of gatifloxacin, grepafloxacin, levofloxacin, moxifloxacin and trovafloxacin against 4151 Gram-negative and Gram-positive organisms. Int. J. Antimicrob. Agents **14:**45–50.
5. **Brooun, A., S. Liu, and K. Lewis.** 2000. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. Antimicrob. Agents Chemother. **44:**640–646.
6. **Cheng, J., J. A. Thanassi, C. L. Thoma, Y. Ou, B. J. Bradbury, M. Deshpande, and M. J. Pucci.** 2006. Abstr. 46th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F1-1995.
7. **Chu, D. T. W., P. B. Fernandes, A. K. Claiborne, L. Shen, and A. G. Pernet.** 1988. Structure-activity relationships in quinolone antibacterials: design, synthesis and biological activities of novel isothiazoloquinolones. Drugs Exp. Clin. Res. **14:**379–383.
8. **Chu, D. T. W., I. M. Lico, A. K. Claiborne, J. J. Plattner, and A. G. Pernet.** 1990. Structure-activity relationship of quinolone antibacterial agents: the effects of C-2 substitution. Drugs Exp. Clin. Res. **16:**215–224.
9. **Clerch, B., E. Rivera, and M. Llagostera.** 1996. Identification of a pKM101 region which confers a slow growth rate and interferes with susceptibility to quinolone in *Escherichia coli* AB1157. J. Bacteriol. **178:**5568–5572.
10. **Cui, L., E. Tominaga, H. M. Neoh, and K. Hiramatsu.** 2006. Correlation between reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. Antimicrob. Agents Chemother. **50:**1079–1082.
11. **Discotto, L. F., L. E. Lawrence, K. L. Denbleyker, and J. F. Barrett.** 2001. *Staphylococcus aureus* mutants selected by BMS-284756. Antimicrob. Agents Chemother. **45:**3273–3275.
12. **Ferrero, L., B. Cameron, and J. Crouzet.** 1995. Analysis of *gyrA* and *grlA* mutations in stepwise-selected ciprofloxacin-resistant mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **39:**1554–1558.
13. **French, G. L.** 2005. Clinical impact and relevance of antibiotic resistance. Adv. Drug Deliv. Rev. **57:**1514–1527.
14. **Furuya, E. Y., and F. D. Lowy.** 2006. Antimicrobial-resistant bacteria in the community setting. Nat. Rev. Microbiol. **4:**36–45.
15. **Gould, I. M.** 2005. The clinical significance of methicillin-resistant Staphylococcus aureus. J. Hosp. Infect. **61:**277–282.
16. **Griggs, D. J., H. Marona, and L. J. Piddock.** 2003. Selection of moxifloxacin-resistant Staphylococcus aureus compared with five other fluoroquinolones. J. Antimicrob. Chemother. **51:**1403–1407.
17. **Hancock, R. E.** 2005. Mechanisms of action of newer antibiotics for Gram-positive pathogens. Lancet Infect. Dis. **5:**209–218.
18. **Heisig, P.** 2001. Inhibitors of bacterial topoisomerases: mechanisms of action and resistance and clinical aspects. Planta Med. **67:**3–12.
19. **Hooper, D. C.** 2002. Fluoroquinolone resistance among Gram-positive cocci. Lancet Infect. Dis. **2:**530–538.
20. **Hooper, D. C.** 2001. Mechanisms of action of antimicrobials: focus on fluoroquinolones. Clin. Infect. Dis. **32:**S9–S15.
21. **Hooper, D. C.** 2000. Mechanisms of action and resistance of older and newer fluoroquinolones. Clin. Infect. Dis. **2:**S24–S28.
22. **Horii, T., Y. Suzuki, A. Monji, M. Morita, H. Muramatsu, Y. Kondo, M. Doi, A. Takeshita, T. Kanno, and M. Maekawa.** 2003. Detection of mutations in quinolone resistance-determining regions in levofloxacin- and methicillin-resistant Staphylococcus aureus: effects of the mutations on fluoroquinolone MICs. Diagn. Microbiol. Infect. Dis. **46:**139–145.
23. **Ince, D., X. Zhang, and D. C. Hooper.** 2003. Activity of and resistance to moxifloxacin in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47:** 1410–1415.
24. **Ince, D., X. Zhang, L. C. Silver, and D. C. Hooper.** 2003. Topoisomerase targeting with and resistance to gemifloxacin in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47:**274–282.
25. **Ince, D., and D. C. Hooper.** 2000. Mechanisms and frequency of resistance to premafloxacin in *Staphylococcus aureus*: novel mutations suggest novel drug-target interactions. Antimicrob. Agents Chemother. **44:**3344–3350.
26. **Jones, R. N., M. L. Beach, M. A. Pfaller, and G. V. Doern.** 1998. Antimicrobial activity of gatifloxacin tested against 1676 strains of ciprofloxacin-resistant gram-positive cocci isolated from patient infections in North and South America. Diagn. Microbiol. Infect. Dis. **32:**247–252.
27. **Low, D. E., M. Muller, C. L. Duncan, B. M. Willey, J. C. de Azavedo, A. McGeer,**

B. N. Kreiswirth, S. Pong-Porter, and D. J. Bast. 2002. Activity of BMS-284756, a novel des-fluoro(6) quinolone, against *Staphylococcus aureus*, including contributions of mutations to quinolone resistance. Antimicrob. Agents Chemother. **46:**1119–1121.
28. **MacGowan, A. P.** 1999. Moxifloxacin (Bay 12-8039): a new methoxy quinolone antibacterial. Expert Opin. Investig. Drugs **8:**181–199.
29. **Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey, D. A. Talan, and EMERGEncy ID Net Study Group.** 2006. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N. Engl. J. Med. **355:**666–674.
30. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 6th ed. M7–A6. National Committee for Clinical Laboratory Standards, Wayne, PA.
31. **Ng, E. Y., M. Trucksis, and D. C. Hooper.** 1996. Quinolone resistance mutations in topoisomerase IV: relationship to the *flqA* locus and genetic evidence that topoisomerase IV is the primary target and DNA gyrase is the secondary target of fluoroquinolones in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40:**1881–1888.
32. **Noguchi, N., T. Okihara, Y. Namiki, Y. Kumaki, Y. Yamanaka, M. Koyama, K. Wakasugi, and M. Sasatsu.** 2005. Susceptibility and resistance genes to fluoroquinolones in methicillin-resistant *Staphylococcus aureus* isolated in 2002. Int. J. Antimicrob. Agents **25:**374–379.
33. **Peeters, M. J., and J. C. Sarria.** 2005. Clinical characteristics of linezolid-resistant Staphylococcus aureus infections. Am. J. Med. Sci. **330:**102–104.
34. **Pucci, M. J., J. Cheng, S. D. Podos, C. L. Thoma, J. A. Thanassi, D. D. Buechter, G. Mushtaq, G. A. Vigliotti, Jr., B. J. Bradbury, and M. Deshpande.** 2007. In vitro and in vivo antibacterial activities of isothiazoloquinolones against resistant gram-positive pathogens. Antimicrob. Agents Chemother. **51:**1259–1267.
35. **Rice, L. B.** 2006. Antimicrobial resistance in gram-positive bacteria. Am. J. Med. **119**(Suppl. 1)**:**S11–S19; discussion, S62–S70.
36. **Roychoudhury, S., and B. Ledoussal.** 2002. Non-fluorinated quinolones (NFQs): new antibacterials with unique properties against quinolone-resistant gram-positive pathogens. Curr. Drug Targets Infect. Disord. **2:**51–65.
37. **Schweon, S. J.** 2006. MRSA extends its reach. RN **69:**33–34,36; quiz, 37.
38. **Shah, P. M.** 2005. The need for new therapeutic agents: what is the pipeline? Clin. Microbiol. Infect. **11**(Suppl. 3)**:**36–42.
39. **Sheldon, A. T., Jr.** 2005. Antibiotic resistance: a survival strategy. Clin. Lab. Sci. **18:**170–180.
40. **Sierra, J. M., J. G. Cabeza, M. Ruiz Chaler, T. Montero, J. Hernandez, J. Mensa, M. Llagostera, and J. Vila.** 2005. The selection of resistance to and the mutagenicity of different fluoroquinolones in Staphylococcus aureus and Streptococcus pneumoniae. Clin. Microbiol. Infect. **11:**750–758.
41. **Skiest, D. J.** 2006. Treatment failure resulting from resistance of *Staphylococcus aureus* to daptomycin. J. Clin. Microbiol. **44:**655–656.
42. **Strahilevitz, J., Y. Onodera, and D. C. Hooper.** 2006. An improved expression plasmid for affinity purification of Staphylococcus aureus gyrase A subunit. Protein Expr. Purif. **47:**10–15.
43. **Strahilevitz, J., A. Robicsek, and D. C. Hooper.** 2006. Role of the extended α4 domain of *Staphylococcus aureus* gyrase A protein in determining low sensitivity to quinolones. Antimicrob. Agents Chemother. **50:**600–606.
44. **Takei, M., H. Fukuda, R. Kishii, and M. Hosaka.** 2001. Target preference of 15 quinolones against Staphylococcus aureus, based on antibacterial activities and target inhibition. Antimicrob. Agents Chemother. **45:**3544–3547.
45. **Trucksis, M., J. S. Wolfson, and D. C. Hooper.** 1991. A novel locus conferring fluoroquinolone resistance in *Staphylococcus aureus*. J. Bacteriol. **173:**5854–5860.
46. **Truong-Bolduc, Q. C., J. Strahilevitz, and D. C. Hooper.** 2006. NorC, a new efflux pump regulated by MgrA of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **50:**1104–1107.
47. **Truong-Bolduc, Q. C., P. M. Dunman, J. Strahilevitz, S. J. Projan, and D. C. Hooper.** 2005. MgrA is a multiple regulator of two new efflux pumps in *Staphylococcus aureus*. J. Bacteriol. **187:**2395–2405.
48. **Truong-Bolduc, Q. C., X. Zhang, and D. C. Hooper.** 2003. Characterization of NorR protein, a multifunctional regulator of *norA* expression in *Staphylococcus aureus*. J. Bacteriol. **185:**3127–3138.
49. **Yamagishi, J.-I., T. Kojima, Y. Oyamada, K. Fujimoto, H. Hattori, S. Nakamura, and M. Inoue.** 1996. Alterations in DNA topoisomerase IV *grlA* gene responsible for quinolone resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40:**1157–1163.
50. **Zhao, X., W. Eisner, N. Perl-Rosenthal, B. Kreiswirth, and K. Drlica.** 2003. Mutant prevention concentration of garenoxacin (BMS-284756) for ciprofloxacin-susceptible or -resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47:**1023–1027.