In Vitro and *In Vivo* Profiles of ACH-702, an Isothiazoloquinolone, against Bacterial Pathogens[▽]

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ACH-702, a novel isothiazoloquinolone (ITQ), was assessed for antibacterial activity against a panel of Gram-positive and Gram-negative clinical isolates and found to possess broad-spectrum activity, especially against antibiotic-resistant Gram-positive strains, including methicillin-resistant *Staphylococcus aureus* (MRSA). For Gram-negative bacteria, ACH-702 showed exceptional potency against *Haemophilus influenzae*, *Moraxella catarrhalis*, and a *Neisseria* sp. but was less active against members of the *Enterobacteriaceae*. Good antibacterial activity was also evident against several anaerobes as well as *Legionella pneumophila* and *Mycoplasma pneumoniae*. Excellent bactericidal activity was observed for ACH-702 against several bacterial pathogens in time-kill assays, and postantibiotic effects (PAEs) of >1 h were evident with both laboratory and clinical strains of staphylococci at 10× MIC and similar in most cases to those observed for moxifloxacin at the same MIC multiple. *In vivo* efficacy was demonstrated against *S. aureus* with murine sepsis and thigh infection models, with decreases in the number of CFU/thigh equal to or greater than those observed after vancomycin treatment. Macromolecular synthesis assays showed specific dose-dependent inhibition of DNA replication in staphylococci, and biochemical analyses indicated potent dual inhibition of two essential DNA replication enzymes: DNA gyrase and topoisomerase IV. Additional biological data in support of an effective dual targeting mechanism of action include the following: low MIC values (≤ 0.25 µg/ml) against staphylococcal strains with single mutations in both *gyrA* and *grlA* (*parC*), retention of good antibacterial activity (MICs of ≤ 0.5 µg/ml) against staphylococcal strains with two mutations in both *gyrA* and *grlA*, and low frequencies for the selection of higher-level resistance ($<10^{-10}$). These promising initial data support further study of isothiazoloquinolones as potential clinical candidates.

Antibiotic resistance creates significant treatment issues for the worldwide health care community in regard to serious bacterial infections (3). Current pathogens of concern include methicillin-resistant *Staphylococcus aureus* (MRSA) (35), penicillin-resistant *Streptococcus pneumoniae* (PRSP) (22, 36), vancomycin-resistant enterococci (VRE) (15), extended-spectrum β-lactamase (ESBL) Gram-negative bacteria (17, 29, 31, 47), and multidrug-resistant *Pseudomonas aeruginosa* (17, 31). Staphylococci, particularly MRSA but also including coagulase-negative strains, have posed a challenge in hospital settings, resulting in substantial morbidity and mortality. Vancomycin is often used to treat MRSA infections, but in recent years there have been reports of vancomycin-nonsusceptible isolates and the reduced effectiveness of this drug (2, 6, 7, 18, 30). In addition, community-acquired MRSA accounts for an increasing number of serious infections (8). Despite the growing unmet medical need, few new antibacterial agents have been introduced in recent years that are effective against many of these often highly resistant clinical isolates (5).

One of our most important classes of antibiotics has been the fluoroquinolones; however, resistance to these drugs has also increased over time. In particular, most MRSA clinical isolates became resistant to fluoroquinolones within 5 years of

their introduction for clinical use (1). Previously, we described a class of compounds with structural similarities to quinolones, the isothiazoloquinolones (ITQs; subset of heteroaryl isothiazolones), which displayed potent and broad-spectrum antibacterial activity against a variety of important pathogens, including fluoroquinolone-resistant isolates (32, 43, 44, 45). Prototype representatives from this class have been synthesized previously (10, 11), but none to date have been successfully developed as antibacterial drugs, for reasons unknown. These compounds were found to be excellent inhibitors of both bacterial DNA gyrase and topoisomerase IV, essential enzymes involved in DNA replication. This potent dual targeting of both enzymes probably accounts for the retention of good antibacterial activity against quinolone-resistant strains with multiple target mutations and the difficulty in obtaining mutants by selection with ITQs (9).

In this work, we describe our optimized lead compound, ACH-702 (Fig. 1), which had the best overall *in vitro* profile in our ITQ analog library in regard to antibacterial, protein binding and target inhibition properties (33, 41). The effectiveness of this compound against Gram-positive isolates, particularly MRSA and including quinolone-resistant strains, is especially attractive. In addition, ACH-702 also exhibits antibacterial activity against many Gram-negative strains, a property that is lacking among most currently marketed drugs used to treat MRSA infections. The mechanism of action involves potent inhibition of two clinically validated bacterial targets, DNA gyrase and topoisomerase IV, and thus suggests a more difficult path for resistance emergence. Therefore, this bactericidal

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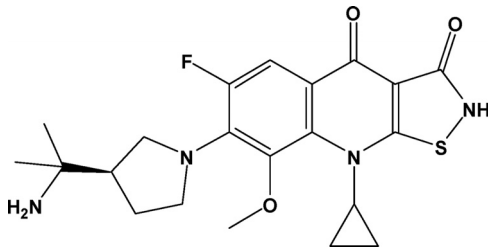† Present address: Rib-X Pharmaceuticals, New Haven, CT 06511.

FIG. 1. Chemical structure of ACH-702.

compound offers the potential for further development as a new antibacterial agent, particularly against antibiotic-resistant Gram-positive pathogens.

MATERIALS AND METHODS

Bacterial strains. Specific strains used in this work are listed in Table 1. All clinical isolates used and listed in Tables 2, 3, and 4, including MRSA strain ACH-0231, were from the strain collection at Eurofins Medinet, Chantilly, VA. Isolates were selected to include important emerging resistance phenotypes. For *Staphylococcus aureus*, this included methicillin-, vancomycin-, and quinolone-resistant, vancomycin intermediate-resistant, and linezolid-nonsusceptible isolates. For the streptococci, macrolide- and penicillin-resistant isolates were included. All other organisms were randomly selected from the Eurofins Medinet collection that contains isolates from clinical sites around the world. MRSA strains NY2746, BSA643, and BSA678 were obtained from Donald Low, Department of Microbiology, Toronto Medical Laboratories and Mount Sinai Hospital, Toronto, Canada (23). MRSA strain BK2384 was obtained from Barry Kreiswirth at the Public Health Research Institute/UMDNJ.

***In vitro* susceptibility testing.** All susceptibility testing was done either at Achillion Pharmaceuticals or Eurofins Medinet in cation-adjusted Mueller-Hinton II broth (CAMHB; media obtained from BD, Sparks, MD, unless otherwise indicated). Streptococci were supplemented with 2 to 5% lysed horse blood, and *Haemophilus influenzae* was tested in *Haemophilus* test medium. Aerobic isolates were tested by broth microdilution using CLSI standards (12), with final inoculum sizes of approximately 5×10^5 CFU/ml for most strains. Inoculated plates were incubated aerobically at 35 to 37°C for 24 h, and the MIC was defined as the minimum concentration of compound that resulted in no visible growth after 24 h at 35 to 37°C. Anaerobic organisms were tested by agar dilution as recommended by CLSI (13) using Brucella agar supplemented with 5 mg/ml hemin, 1 mg/ml vitamin K, and 5% laked sheep blood. Inocula consisted of colonies taken from 24-h growth on enriched blood agar and resuspended to approximate the turbidity of a 0.5 McFarland standard in Brucella broth. Plates were incubated under anaerobic conditions at 35 to 37°C for 48 h. The MIC_{50} and MIC_{90} values were calculated by standard methods at Eurofins Medinet.

Time-kill studies. Time-kill studies were done as described previously (4, 32). Briefly, all strains were cultured overnight at 35 to 37°C in CAMHB, and cells were diluted into fresh medium, grown to exponential phase, and then diluted again in medium to adjust cell densities to approximately 10^7 CFU/ml. Compounds were then added at concentration multiples of the MIC. Rates of killing were determined by measuring the reduction in viable bacteria ($\log_{10}$ CFU/ml) at 0, 1, 2, 4, 6, and 24 h at fixed concentrations of compound. Experiments were performed in duplicate. If plates contained fewer than 10 CFU/ml, the number of colonies was considered to be below the limit of quantitation. Samples of culture containing compound were diluted at least 10-fold to minimize drug carryover to the CAMHB plates.

PAE. The postantibiotic effect (PAE) was determined following treatment of *S. aureus* with compound at 10× MIC for 1 h (16). Prewarmed 10-ml CAMHB cultures with or without antibiotic were inoculated with approximately 2 to 4 × 10^7 CFU/ml *S. aureus* cells in logarithmic growth phase, and then grown with shaking at 35°C to 37°C for 1 h. After this exposure period, cultures were diluted 1:1,000 in prewarmed CAMHB to initiate recovery. Viable counts were measured prior to exposure, immediately after dilution, and hourly for 4 to 8 h of recovery, by plating appropriate dilutions onto CAMHB agar. An additional control with untreated bacteria was diluted in media plus compound at 0.01× MIC to establish that the residual compound during recovery had no effect on growth and viability (data not shown). The PAE was defined as $T - C$, where T is the time required for viability counts of an antibiotic-exposed culture to increase 1 $\log_{10}$ above the counts present immediately after dilution and C is the corresponding time for the growth control without antibiotic (28).

Macromolecular synthesis assay. The effects of ACH-702 and control compounds ciprofloxacin (DNA synthesis), rifampin (RNA synthesis), and chloramphenicol (protein synthesis) on DNA, RNA, and protein synthesis in bacteria were measured using radiolabeled precursors [^{3}H]thymidine (DNA), [^{3}H]uracil (RNA), and [^{3}H]leucine (protein) in mid-exponential-phase cultures (10^8 CFU/ml) of *S. aureus* ATCC 29213 in chemically defined medium (40) as previously

TABLE 1. Bacterial strains and relevant genotypes[a]

Organism	Strain	Genotype[b] or description	Source and/or reference
S. aureus	ATCC 29213	CLSI QC reference strain; MSSA	ATCC
	ATCC 13709	Laboratory standard strain; strain Smith	ATCC
	ATCC 33591	Laboratory standard strain; MRSA	ATCC
	ACH-0204	ATCC 29213 *gyrA(Ser84Leu)*	9
	ACH-0216	ATCC 29213 *grlA(Ser80Phe)*	9
	ACH-0126	ATCC 29213 *gyrA(Ser84Leu) grlA(Ser80Phe)*	9
	ACH-0130	ATCC 29213 *gyrA(Ser84Leu Glu88Val) grlA(Ser80Phe Ala116Val)*	9
	COL	MRSA clinical isolate	26
	BK2384	Cip^r, Tmp^r, Tet^r, Ery^r; HA-MRSA *gyrA(Ser84Leu) grlA(Ser80Phe)*	B. Kreiswirth, PHRI/UMDNJ
	ATCC 700699	*mecA$^+$ gyrA(Ser84Leu Glu409Lys) grlA(Ser80Phe)*; Mu50; VISA; MRSA	ATCC (18)
	NY2746	*mecA$^+$ gyrA(Ser84Leu) grlA(Ser80Phe)*; MRSA	23
	BSA643	*mecA$^+$ gyrA(Ser84Leu) grlA(Ser80Tyr Glu84Gly)*; MRSA	23
	BSA678	*mecA$^+$ gyrA(Ser84Leu Ser85Pro) grlA(Ser80Phe Glu84Lys)*; MRSA	23
	ACH-0231	*mecA$^+$ gyrA(Ser84Leu Glu88Lys) grlA(Ser80Tyr Glu84Gly)*; MRSA	Eurofins Medinet, Chantilly, VA
E. faecalis	ATCC 29212	CLSI QC reference strain	ATCC
E. coli	ATCC 25922	CLSI QC reference strain	ATCC
P. aeruginosa	ATCC 27853	CLSI QC reference strain	ATCC

[a] Abbreviations: Cip^r, ciprofloxacin resistant; Tmp^r, trimethoprim resistant; Tet^r resistant, tetracycline; Ery^r, erythromycin resistant; CLSI, Clinical and Laboratory Standards Institute, Wayne, PA; ATCC, American Type Culture Collection, Manassas, VA; HA-MRSA, hospital-associated MRSA.

[b] *mecA* is the gene encoding PBP2a; *gyrA* and *grlA* are the genes encoding the A subunit of gyrase and topoisomerase IV, respectively.

TABLE 2. ACH-702 antibacterial activity against Gram-positive clinical isolates

Organism (no. of isolates)[b]	Antimicrobial	MIC (µg/ml)[a]		
		Range	MIC_{50}	MIC_{90}
Staphylococcus aureus (82)				
MSSA, all isolates (12)	ACH-702	0.015–1	0.03	0.25
	Levofloxacin	0.12–>16	0.25	>16
	Oxacillin	0.25–0.5	0.5	0.5
	Linezolid	2–2	2	2
	Vancomycin	1–2	1	2
MRSA, all isolates (70)	ACH-702	0.008–0.5	0.06	0.25
	Levofloxacin	0.12–>16	16	>16
	Oxacillin	4–>16	>16	>16
	Linezolid	1–2	1	2
	Vancomycin	0.5–2	1	1
MRSA, FQ[NS] (49)	ACH-702	0.03–0.5	0.06	0.25
	Levofloxacin	2–>16	16	>16
	Oxacillin	4–>16	>16	>16
	Linezolid	1–2	2	2
	Vancomycin	0.5–2	1	1
Staphylococcus epidermidis				
All isolates (21)	ACH-702	0.015–0.25	0.03	0.25
	Levofloxacin	0.12–>16	0.25	>16
	Oxacillin	0.25–>16	1	>16
	Linezolid	0.5–1	1	1
	Vancomycin	1–2	2	2
FQ[NS] (9)	ACH-702	0.12–0.25	0.12	0.25
	Levofloxacin	8–>16	16	>16
	Oxacillin	0.25–>16	8	>16
	Linezolid	0.5–1	1	1
	Vancomycin	1–2	2	2
Streptococcus pneumoniae				
All isolates (31)	ACH-702	0.015–0.06	0.03	0.06
	Levofloxacin	0.5–2	1	1
	Ceftriaxone	0.015–8	0.5	1
	Linezolid	0.5–2	1	1
	Penicillin	<0.015–8	1	4
PEN[NS] (18)	ACH-702	0.03–0.06	0.03	0.06
	Levofloxacin	0.5–2	1	2
	Ceftriaxone	0.5–8	1	2
	Linezolid	0.5–2	1	1
	Penicillin	0.25–8	4	4
Enterococcus faecalis				
All isolates (15)	ACH-702	0.06–0.5	0.25	0.5
	Levofloxacin	0.5–>16	>16	>16
	Linezolid	0.5–>16	2	>16
	Vancomycin	0.25–>32	1	>32
FQ[NS] (9)	ACH-702	1–2	1	1
	Levofloxacin	16–>16	>16	>16
	Linezolid	0.5–>16	2	>16
	Vancomycin	1–>32	1	>32
Enterococcus faecium (14)[c]	ACH-702	0.25–4	1	2
	Levofloxacin	4–>16	>16	>16
	Linezolid	0.5–>16	2	2
	Vancomycin	0.25–>32	>32	>32

[a] For current CLSI breakpoints for comparators, see reference 14.
[b] FQ[NS], fluoroquinolone-nonsusceptible isolates as indicated by levofloxacin MICs relative to CLSI breakpoints; PEN[NS], penicillin-nonsusceptible isolates, including intermediate (I) and resistant (R) isolates.
[c] All *E. faecium* isolates tested were fluoroquinolone resistant.

TABLE 3. ACH-702 antibacterial activity against Gram-negative clinical isolates

Organism (no. of isolates)[b]	Antimicrobial	MIC (μg/ml)[a]		
		Range	MIC$_{50}$	MIC$_{90}$
Enterobacteriaceae, all isolates (120)				
Escherichia coli (30)	ACH-702	0.06–16	0.12	8
	Ciprofloxacin	0.015–>64	0.03	32
	Moxifloxacin	0.06–32	0.06	16
	Ceftazidime	≤0.03–>16	0.25	0.25
	Imipenem	0.12–0.25	0.25	0.25
	Gentamicin	≤0.12–>8	0.5	>8
Enterobacter cloacae (30)	ACH-702	0.06–32	0.25	8
	Ciprofloxacin	0.015–>64	0.03	16
	Moxifloxacin	0.06–>64	0.12	16
	Ceftazidime	≤0.03–>16	0.25	>16
	Imipenem	0.06–2	0.5	1
	Gentamicin	≤0.12–>8	0.25	>8
Klebsiella pneumoniae (30)	ACH-702	0.06–64	0.25	2
	Ciprofloxacin	0.015–>64	0.06	1
	Moxifloxacin	0.03–>64	0.12	2
	Ceftazidime	≤0.03–>16	0.12	>16
	Imipenem	0.12–8	0.25	0.5
	Gentamicin	≤0.12–>8	0.25	0.5
Proteus mirabilis (30)	ACH-702	0.06–16	0.25	4
	Ciprofloxacin	0.03–>64	0.06	32
	Moxifloxacin	0.25–>64	0.5	64
	Ceftazidime	≤0.03–0.12	0.06	0.06
	Imipenem	0.06–4	2	4
	Gentamicin	0.5–>8	1	>8
Enterobacteriaceae FQ[NS] (22)	ACH-702	0.5–64	8	16
	Ciprofloxacin	2–>64	32	>64
	Moxifloxacin	4–>64	16	>64
	Ceftazidime	0.06–>16	16	>16
	Imipenem	0.06–8	0.5	4
	Gentamicin	0.25–>8	1	>8
Nonfermenters, all isolates (60)				
Acinetobacter baumannii (30)	ACH-702	0.06–8	1	4
	Ciprofloxacin	0.15–>64	32	>64
	Moxifloxacin	0.03–64	4	32
	Ceftazidime	0.25–>16	4	>16
	Imipenem	0.12–>8	0.5	>8
	Gentamicin	≤0.12–>8	1	>8
A. baumannii FQ[NS] (16)	ACH-702	1–8	2	8
	Ciprofloxacin	32–>64	64	>64
	Moxifloxacin	4–64	16	32
	Ceftazidime	2–>16	>16	>16
	Imipenem	0.12–>8	8	>8
	Gentamicin	0.25–>8	>8	>8
Pseudomonas aeruginosa (30)	ACH-702	0.12–32	1	8
	Ciprofloxacin	0.03–32	0.25	8
	Moxifloxacin	0.12–64	2	64
	Ceftazidime	≤0.03–>16	2	8
	Imipenem	0.5–>8	2	4
	Gentamicin	0.25–>8	1	2
P. aeruginosa FQ[NS] (11)	ACH-702	1–32	8	32
	Ciprofloxacin	2–32	8	32
	Moxifloxacin	4–64	64	64
	Ceftazidime	0.5–>16	2	16
	Imipenem	0.5–>8	2	8
	Gentamicin	0.25–>8	2	8

TABLE 3—*Continued*

Organism (no. of isolates)[b]	Antimicrobial	MIC (μg/ml)[a]		
		Range	MIC$_{50}$	MIC$_{90}$
Respiratory Gram-negatives (23)				
Haemophilus influenzae (10)	ACH-702	0.06–0.12	0.06	0.12
	Levofloxacin	0.015–0.06	0.015	0.03
	Ampicillin	0.12–>16	>16	>16
	Ceftriaxone	0.015–0.03	0.015	0.015
Moraxella catarrhalis (13)	ACH-702	0.03–0.12	0.06	0.06
	Levofloxacin	0.03–0.06	0.03	0.06
	Ampicillin	1–16	4	8
	Ceftriaxone	0.03–2	1	2

[a] For current CLSI breakpoints for comparators, see reference 14.
[b] FQ[NS], fluoroquinolone-nonsusceptible isolates as defined by ciprofloxacin MICs relative to CLSI breakpoints.

described (27). Final concentrations of 5 μCi/ml for thymidine and 2.5 μCi/ml for each of the other precursors were added to cultures immediately before the addition of antibiotics (10× MIC). Negative controls for the macromolecular assays consisted of all reaction materials with no antibiotics added. The resulting counts were used as the 100% values. After an additional 20-min incubation at 37°C in the presence of antibiotics, samples were removed for trichloroacetic acid precipitation and subsequent scintillation counter analyses for the determination of radioactive incorporation into DNA, RNA, or protein, and the data were expressed as the percent inhibition of incorporation into a drug-free control.

Selection of resistant mutants. *S. aureus* parent strains, ATCC 29213 or ATCC 33591, were cultured in brain heart infusion (BHI) broth overnight and concentrated in a 1/10 volume of phosphate-buffered saline, and approximately 1×10^{10} CFU were plated onto BHI agar plates containing 1×, 2×, 4× and 8× MIC concentrations of compounds and incubated at 37°C for 48 to 72 h as described previously (9). Selection frequencies of resistant mutants were calculated as the ratios of resistant mutants to untreated control cells. All resistant mutants were confirmed by MIC and DNA sequencing of the quinolone resistance determining regions (QRDRs) (46) of *gyrA* and *grlA*.

DNA sequencing. Template genomic DNAs were prepared by boiling one fresh *S. aureus* colony in 50 μl of distilled water and were subjected to PCR amplification. ABI 3100 DNA sequencers used PCR DNAs as templates for automated sequencing at W. M. Keck Facility, Yale University, New Haven, CT.

Cloning, protein expression, and purification of *S. aureus* enzymes. Expression of wild-type proteins GyrA, GyrB, GrlA (ParC), and GrlB (ParE) and mutant proteins GyrA(Ser84Leu), GrlA(Ser80Phe), GyrA(Ser84Leu Glu88Lys), and GrlA(Ser80Phe Glu84Lys) was performed as previously described (9, 39), with *S. aureus* COL as the source for wild-type genes (26). Briefly, cloning was done using pET vector overexpression constructs with 6His tags (Novagen, EMD, La Jolla, CA). The GyrA constructs additionally encoded the cleavage site for tobacco etch virus (TEV) protease downstream of the thioredoxin gene for enhanced solubility (39). All mutant constructs were made by site-directed mutagenesis using the QuikChange II XL kit (Stragagene, Santa Clara, CA) according to the manufacturer's instructions. All 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 chromatography. GyrA protein was cleaved from thioredoxin with recombinant TEV protease (Invitrogen, Carlsbad, CA) during purification.

***S. aureus* topoisomerase IV and DNA gyrase assays.** Topoisomerase IV proteins were reconstituted *in vitro* as wild-type enzyme (GrlA[wild type] plus GrlB) or mutant enzyme (GrlA[mutant] plus GrlB) at subunit molar ratios of 1:1. Topoisomerase IV activity was measured by a decatenation assay that monitored the ATP-dependent unlinking of DNA minicircles from kinetoplast DNA (Topogen, Inc., Port Orange, FL). DNA gyrase proteins were reconstituted *in vitro* as wild-type enzyme (GyrA[wild type] plus GyrB) or mutant enzyme (GyrA[mutant] plus GyrB) at subunit molar ratios of 1:1. Gyrase activity was measured by a supercoiling assay that monitored the ATP-dependent conversion of relaxed pBR322 DNA to the supercoiled form (Topogen). The products of both enzymes were quantified by ethidium bromide fluorescence following agarose gel electro-

TABLE 4. ACH-702 antibacterial activity against anaerobic clinical isolates

Organism (no. of isolates)	Antimicrobial	MIC (μg/ml)[a]		
		Range	MIC$_{50}$	MIC$_{90}$
Bacteroides fragilis (10)	ACH-702	0.06–0.12	0.12	0.12
	Clindamycin	0.25–4	1	4
	Imipenem	0.25–0.5	0.25	0.25
	Penicillin	0.5–32	16	16
Clostridium difficile (10)	ACH-702	0.25–4	0.25	4
	Clindamycin	1–>32	2	>32
	Imipenem	4–16	4	4
	Penicillin	0.5–4	1	1
Propionibacterium acnes (10)	ACH-702	0.03–0.06	0.06	0.06
	Clindamycin	≤0.03–0.06	0.06	0.06
	Imipenem	0.03–0.12	0.06	0.06
	Penicillin	≤0.12–≤0.12	≤0.12	≤0.12
Peptostreptococcus sp. (10)	ACH-702	0.008–0.12	0.015	0.06
	Clindamycin	0.12–4	0.25	1
	Imipenem	0.03–0.5	0.06	0.25
	Penicillin	≤0.25–0.5	0.25	0.5

[a] For current CLSI breakpoints for comparators, see reference 14.

phoresis. IC_{50}s for both enzymes were defined as the concentration of compound that reduced product formation to 50% of the uninhibited control. All values were the result of at least two independent experiments. Compound potencies were compared with values determined for quinolones such as moxifloxacin.

In vivo **efficacy against experimental infections. (i) Mouse thigh muscle infection.** Adult female CD-1 mice (19 to 28 g; Charles River Laboratories, Wilmington, MA) were made neutropenic with two intraperitoneal injections of cyclophosphamide (Sigma; 150 mg/kg of body weight in 10 ml/kg) at 4 days and 1 day before infection. Groups of three mice each were infected intramuscularly with approximately 10^5 CFU of MRSA ATCC 33591 and were treated with a single subcutaneous dose of compound at 2 h postinfection. A control group of infected animals was not treated. Thighs from three animals were harvested at 2, 4, 6, or 24 h after treatment initiation. The thighs were processed by aseptic removal from each animal, dilution into sterile phosphate-buffered saline, and homogenization with a tissue homogenizer. Serial dilutions of the tissue homogenates were spread on Trypticase-soy agar (TSA; BD) plates, and the plates were incubated at 37°C overnight. The CFU/thigh values were determined from colony counts and compared with the control number of CFU/thigh at the time of treatment.

(ii) Mouse systemic infection. Adult female CD-1 mice (20 to 29 g; Charles River Laboratories, Wilmington, MA) were inoculated intraperitoneally with a sufficient number of pathogens to kill 100% of the untreated animals within 5 days. Each mouse received $\sim$3.9 $\times$ 10^7 CFU/ml of *S. aureus* 13709 suspended in 5% (wt/vol) sterile hog gastric mucin in a volume of 0.5 ml. Test articles were administered at 1 h after pathogen inoculation. The number of mice that survived in each experimental group was monitored up to 5 days after pathogen inoculation, and the doses for 50% of the population of drug-treated animals that survived (50% protective doses [PD_{50}s]) were determined. Each experimental group consisted of six animals, and a minimum of four different concentrations of compound was evaluated. The range of doses was 0.03 to 2 mg/kg of body weight.

Pharmacokinetics in mice. Pharmacokinetic parameters were obtained as previously described (32). Briefly, plasma samples were collected at various time points, and the levels of ACH-702 were quantified by liquid chromatography-mass spectrometry (LC-MS/MS) using an internal standard. Resulting mean plasma concentrations were analyzed by noncompartmental methods with Win-Nonlin (Pharsight, Mountain View, CA). The area under the concentration (AUC)-time curve from time zero to the time of the last quantifiable plasma concentration (AUC_{last}) was calculated using the linear trapezoidal rule. Plasma half life ($t_{1/2}$) and time at which maximum concentration occurs after dosing (t_{max}) were calculated with WinNonlin.

RESULTS

Antibacterial activities against clinical isolates. Table 2 compares the antibacterial activities of ACH-702 against Gram-positive clinical isolates to those of comparator drugs. Against methicillin-susceptible *S. aureus* (MSSA; 12 strains), ACH-702 possessed an MIC_{50} of 0.03 μg/ml and an MIC_{90} of 0.25 μg/ml. For MRSA (70 strains), good antibacterial activity was retained, with an MIC_{50} of 0.06 and an MIC_{90} of 0.25, while the comparator quinolone, levofloxacin, was essentially ineffective, as 49/70 strains (70%) had MICs of >2 μg/ml. The MIC_{90}s of all currently marketed fluoroquinolones for quinolone-resistant MRSA are >4 μg/ml (4). Against those MRSA strains with reduced vancomycin susceptibility, including both intermediate-resistant (VISA) and resistant (VRSA) strains ($n = 8$), ACH-702 also displayed activity essentially equivalent to that against vancomycin-sensitive strains (data not shown). In addition, much greater activity was observed for ACH-702 than for comparator drugs against *Staphylococcus epidermidis* (Table 2), particularly against quinolone-nonsusceptible isolates.

The MIC_{50}s and MIC_{90}s for ACH-702 against *Streptococcus pneumoniae* ($n = 31$; Table 2), including penicillin-resistant isolates, were excellent, with no significant differences observed between penicillin-sensitive (PSSP; 13 strains), penicillin-intermediate (PISP; 3 strains), and penicillin-resistant (PRSP; 15 strains)

phenotypes. The MICs for ACH-702 against penicillin nonsusceptible strains were lower than for penicillin and ceftriaxone, as the MIC_{90} remained at 0.06 μg/ml versus 1 and 2 μg/ml for ceftriaxone and penicillin, respectively. Against enterococcal strains, ACH-702 displayed MIC_{50} and MIC_{90} values of 0.25 and 0.5 μg/ml for *Enterococcus faecalis*. These values were lower than those for all comparators tested for *E. faecalis*. ACH-702 MIC_{50} and MIC_{90} values were 1 μg/ml for the quinolone-nonsusceptible *E. faecalis* isolates (9 strains), contrasting sharply with those for levofloxacin, for which all MICs for quinolone-nonsusceptible *E. faecalis* isolates were ≥16 μg/ml. However, ACH-702 was less active against *E. faecium*, with an MIC_{50} of 1 and an MIC_{90} of 2 μg/ml, similar to those for the linezolid comparator. Against additional streptococci, including *Staphylococcus pyogenes* and viridans group streptococci, ACH-702 was also quite active, with all MIC_{50}s and MIC_{90}s ≤0.03 μg/ml (data not shown).

Table 3 compares the antibacterial activities of ACH-702 against Gram-negative clinical isolates to those of several comparator drugs. Although MICs were not as impressive as those seen for Gram-positive strains, antibacterial activity comparable to those of ciprofloxacin and moxifloxacin was found for four *Enterobacteriaceae*: *E. coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Proteus mirabilis*, as well as *P. aeruginosa*. For *Acinetobacter baumannii*, however, ACH-702 was much more active than the two fluoroquinolone comparators, with MIC_{50} and MIC_{90} values of 1 and 4 μg/ml, respectively. However, against quinolone-nonsusceptible isolates of these species, ACH-702 possessed lower MIC_{50}s and MIC_{90}s than ciprofloxacin and moxifloxacin except for *P. aeruginosa*, for which ciprofloxacin was slightly more active (Table 3). For *Haemophilus influenzae* and *Moraxella catarrhalis*, ACH-702 was quite active, with MIC_{90}s ranging from 0.06 to 0.12 μg/ml, including several ampicillin-resistant strains (Table 3). In addition, ACH-702 was tested against both *Neisseria gonorrhea* and *Neisseria meningitidis*, with MICs of ≤0.015 μg/ml for all isolates tested ($n = 10$ for each; data not shown).

MIC values for ACH-702 against four anaerobes are listed in Table 4. Against *Bacteroides fragilis*, MICs ranged from 0.06 to 0.12 μg/ml for all 10 strains tested. These values are slightly lower than those for imipenem and substantially lower than those for penicillin and clindamycin. For *Clostridium difficile*, the MIC_{50} and MIC_{90} were 0.25 and 4 μg/ml, respectively. MIC_{90} values were 0.06 μg/ml against both *Propionibacterium acnes* and *Peptostreptococcus* sp., equal to or better than those for the tested comparator drugs. ACH-702 also displayed good activity against *Legionella pneumophila* and *Mycoplasma pneumoniae*, with MIC_{90}s of 0.015 μg/ml for both organisms (data not shown).

Bactericidal activity. Bactericidal activity for ACH-702 was assessed using two Gram-positive and two Gram-negative strains treated in exponential growth phase. Figure 2A shows representative time-kill curves against an MRSA strain, *S. aureus* ATCC 33591, with vancomycin and moxifloxacin as comparators. Maximum killing was observed at concentrations of 4× MIC and higher, with a 3-log drop in the number of CFU/ml occurring by 2 h after compound addition. In comparison, killing was less rapid with 4× MIC concentrations of moxifloxacin and, especially, vancomycin. Killing was also less rapid at 2× MIC of ACH-702, for which about 4 h was re-
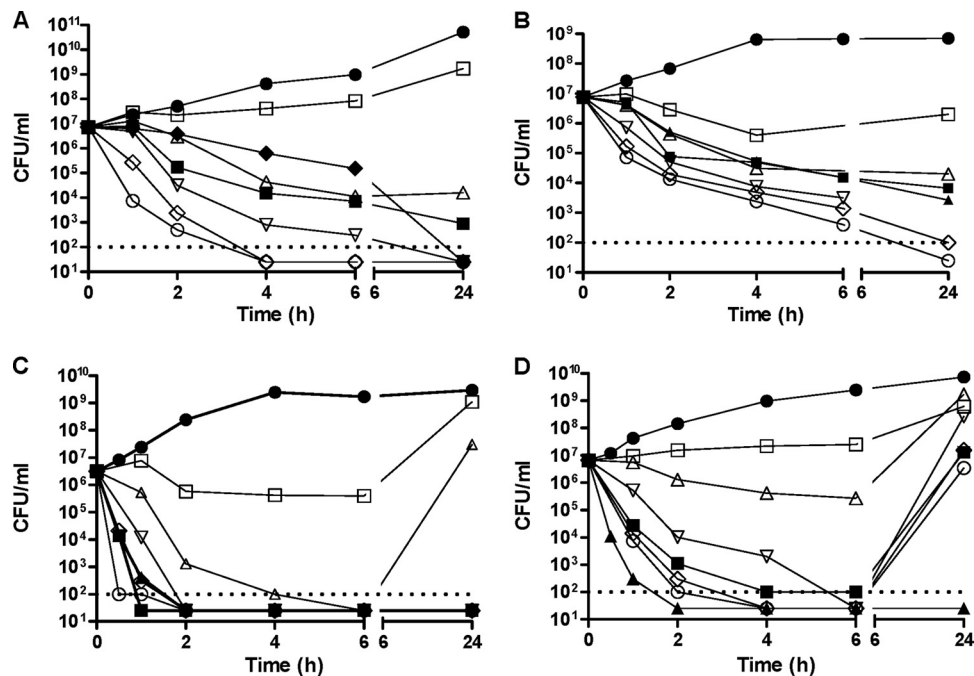
FIG. 2. Time-kill curves for bacterial pathogens. Compounds were added to cultures at time zero, and samples were processed as described in Materials and Methods. Bacterial strains: *S. aureus* ATCC 33591 (A), *E. faecalis* ATCC 29212 (B), *E. coli* ATCC 25922 (C), and *P. aeruginosa* ATCC 27853 (D). Symbols: filled circles, untreated control; filled diamonds, vancomycin (4× MIC); filled squares, moxifloxacin (4× MIC); filled triangles, ciprofloxacin (4× MIC); open squares, ACH-702 (0.5× MIC); open triangles, ACH-702 (1× MIC); open inverted triangles, ACH-702 (2× MIC); open diamonds, ACH-702 (4× MIC); open circles, ACH-702 (8× MIC). The dotted lines indicate the lower limit of quantification; points below the dotted lines could not be assigned precise values for the number of CFU/ml.

quired to cause a 3-log drop in the number of CFU/ml, suggesting a dose-dependent effect on staphylococcal killing up to a concentration equal to 4× MIC. Figure 2B shows bactericidal activity against *E. faecalis* ATCC 29212, for which again a 3-log drop in the number of CFU/ml was observed in approximately 2 h at ≥4× MIC, in contrast to the 6 h required by moxifloxacin and ciprofloxacin at 4× MIC. Against *E. coli* ATCC 25922, a 3-log drop in the number of CFU/ml was observed in less than 2 h at doses of ACH-702 ≥1× MIC as well as for ciprofloxacin and moxifloxacin at 4× MIC (Fig. 2C). For *Pseudomonas aeruginosa* ATCC 27853, a 3-log drop in the number of CFU/ml was observed for ACH-702 within 2 h at doses of ≥2× MIC (Fig. 2D). This was true for moxifloxacin and ciprofloxacin at 4× MIC. However, unexpectedly, a "rebound" effect was observed for moxifloxacin at 4× MIC and at all doses of ACH-702, including 8× MIC, at which cultures recovered to initial densities by 24 h. A rebound effect was also observed for *E. coli* at or below the MIC (Fig. 2C).

PAEs. The postantibiotic effect (PAE) is a measure of the effects of an antibiotic on the cellular viability and growth of bacteria after exposure to that antibiotic is terminated. The PAE of ACH-702 for staphylococci was assessed with four MRSA strains, ATCC 33591, ATCC 700699, BSA678, and BK2384, by measuring the time required for recovery after a defined treatment period (16). PAE results for these strains at 10× MIC were 1.2 to 4.6 h, similar to values obtained with moxifloxacin (data not shown).

Antibacterial activity against quinolone-resistant MRSA clinical isolates and laboratory-selected mutant strains. The

antibacterial activity of ACH-702 was determined against six quinolone-resistant MRSA clinical isolates with characterized target mutations (Table 5). The three marketed quinolones tested as comparators, moxifloxacin, gemifloxacin, and ciprofloxacin, were minimally active or inactive against the six strains that possessed two to four mutations in the QRDRs of *gyrA* and *grlA*. Interestingly, all six strains, including four with possible efflux contributions as indicated by ethidium bromide resistance, were susceptible to ACH-702, as the MICs remained at ≤0.5 μg/ml. Particularly noteworthy, ACH-702 retained good antibacterial activity against strains BSA678 and ACH-0231, for which quinolone MICs were all ≥64 μg/ml. Against quinolone-resistant laboratory mutants (9), differences in MIC values for ACH-702 versus moxifloxacin, gemifloxacin, and ciprofloxacin for strains possessing mutations in *gyrA* and/or *grlA* were evident, with ACH-702 retaining low MICs for strains with multiple QRDR mutations (Table 5).

Activities of ACH-702 against *in vitro*-purified wild-type, single mutant, and double mutant DNA topoisomerase IV and gyrase. To examine bacterial target enzyme inhibition by ACH-702, staphylococcal gyrase and topoisomerase IV enzymes were cloned, expressed, and purified for *in vitro* biochemical studies. In addition, mutant enzyme proteins were engineered via site-directed mutagenesis to introduce the GyrA(Ser84Leu) single and GyrA(Ser84Leu Glu88Lys) double mutations and the GrlA(Ser80Phe) single and GrlA(Ser80Phe Glu84Lys) double mutations that result in increased resistance to fluoroquinolones (19). Enzyme inhibition assays showed that IC_{50}s of the comparator quinolones, moxifloxacin, gemifloxacin, and cipro-

TABLE 5. MICs and target genotypes for laboratory strains and clinical isolates of *S. aureus*

Isolate	ACH-702 MIC (μg/ml)	MIC (μg/ml)				QRDR mutations[a]	
		MXF[b]	GEM	CIP	EtBr	*gyrA*	*grlA*
Clinical							
ATCC 29213	0.004	0.06	0.03	0.25	4	None	None
BK2384	0.08	2	1	64	ND	Ser84Leu	Ser80Phe
NY2746	0.25	8	32	256	16	Ser84Leu	Ser80Phe
ATCC 700699	0.125	4	4	64	>64	Ser84Leu Glu409Lys	Ser80Phe
BSA643	0.25	16	32	256	16	Ser84Leu	Ser80Tyr Glu84Gly
BSA678	0.25	>64	64	128	4	Ser84Leu Ser85Pro	Ser80Phe Glu84Lys
ACH-0231	0.5	64	>64	>64	16	Ser84Leu Glu88Lys	Ser80Tyr Glu84Gly
Laboratory[c]							
ACH-0204	0.016	0.125	0.125	0.5	4	Ser84Leu	None
ACH-0216	0.008	0.25	0.125	2	4	None	Ser80Phe
ACH-0126	0.125	4	4	64	4	Ser84Leu	Ser80Phe
ACH-0130	0.5	32	32	256	32	Ser84Leu Glu88Val	Ser80Phe Ala116Val

[a] QRDR, quinolone resistance determining region. No mutations found in *gyrB* or *grlB*.
[b] Abbreviations: MXF, moxifloxacin; GEM, gemifloxacin; CIP, ciprofloxacin; EtBr, ethidium bromide; ND, not determined.
[c] Parent strain is *S. aureus* ATCC 29213.

floxacin, for wild-type topoisomerase IV decatenation activity were 1.0, 0.4, and 3.0 μM, respectively. The IC_{50}s were consistent with previous reports that quinolones have high binding affinities and thus potently inhibit topoisomerase IV (20, 21). The IC_{50} of ACH-702 was 0.12 μM, more than 3-fold lower than that of gemifloxacin at 0.4 μM, which was reported as dual targeting and one of the most potent quinolones against topoisomerase IV in staphylococci (21). The IC_{50}s of the comparator quinolones for wild-type gyrase supercoiling activity were 7.7, 5.6, and 62 μM for moxifloxacin, gemifloxacin, and ciprofloxacin, respectively, while the IC_{50} of ACH-702 was 0.68 μM, more than 8-fold lower than that of gemifloxacin, the most potent marketed quinolone inhibitor of staphylococcal gyrase to date (21). These data demonstrate that ACH-702 significantly inhibits both topoisomerases in wild-type *S. aureus*, and this balanced activity, with lower IC_{50}s against both enzymes compared with that of gemifloxacin, could account for the lower MIC of ACH-702 (0.004 μg/ml) against wild-type *S. aureus* than that obtained with gemifloxacin (0.03 μg/ml).

A similar analysis was made using gyrase and topoisomerase enzymes with single amino acid substitutions that result in decreased inhibition by quinolones. The IC_{50}s of ACH-702 and comparator quinolones were obtained against mutant topoisomerase IV containing the GrlA(Ser80Phe) subunit and against mutant gyrase containing the GyrA(Ser84Leu) subunit. As shown in Table 6, moxifloxacin, gemifloxacin, and cipro-

floxacin each lost between 12- and 25-fold activity against the mutant topoisomerase IV (IC_{50} = 11.9, 9.8, and 57 μM, respectively). In comparison, the IC_{50} of ACH-702 was 0.6 μM, which is only 5-fold greater than that against the wild-type enzyme and is 16- to 19-fold lower than that with moxifloxacin and gemifloxacin and in the same range as those displayed by the two quinolones against wild-type topoisomerase IV (1.0 and 0.4 μM, respectively). Results were even more pronounced for the mutant gyrase, as the comparator quinolones moxifloxacin, gemifloxacin, and ciprofloxacin showed complete inactivity in the assay (IC_{50}s of >300 μM), while ACH-702 had an IC_{50} of 4.2 μM, which is only 6-fold greater than that against wild-type gyrase and in the same range as those of moxifloxacin and gemifloxacin against wild-type gyrase (7.7 and 5.6 μM, respectively).

Additionally, the activity of ACH-702 and comparators were assessed against enzymes with double mutations that resulted in further resistance to inhibition by quinolones. The IC_{50}s of ACH-702 and comparator quinolones against double mutant topoisomerase IV composed of GrlA(Ser80Phe Glu84Lys) and GrlB(wild type) subunits and against mutant gyrase composed of GyrA(Ser84Leu Glu88Lys) and GyrB(wild type) subunits were obtained (Table 6). Moxifloxacin and gemifloxacin were essentially inactive against both, with IC_{50} values of >200 and >500 μM against double mutant topoisomerase IV and gyrase, respectively. In comparison, the IC_{50}s of ACH-702 were 32 and

TABLE 6. Inhibition of wild-type (WT) and mutant *S. aureus* gyrase and topoisomerase IV catalytic activities by ACH-702 and quinolones[c]

Antimicrobial	IC_{50} (μM)					
	Topoisomerase IV[a]			Gyrase[b]		
	WT	Ser80Phe	Ser80Phe Glu84Lys	WT	Ser84Leu	Ser84Leu Glu88Lys
ACH-702	0.12	0.6	32	0.68	4.2	70
Moxifloxacin	1.0	11.9	>200	7.7	>300	>500
Gemifloxacin	0.4	9.8	>200	5.6	>300	>500
Ciprofloxacin	3.0	57	>200	62	>300	ND

[a] Topoisomerase IV decatenation activity.
[b] Gyrase supercoiling activity. ND, not determined.
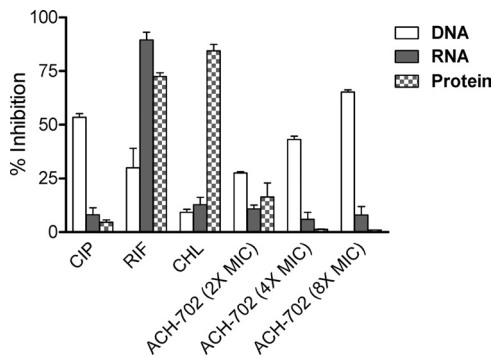[c] Mutant enzymes made via site-directed mutagenesis of WT gene.

FIG. 3. Macromolecular synthesis inhibition in *Staphylococcus aureus*. The assays measured incorporation of radiolabeled precursors into DNA, RNA, or protein as described in Materials and Methods. The percent inhibition represents decrease from untreated controls. Positive control compounds: DNA, ciprofloxacin (CIP); RNA, rifampin (RIF); protein, chloramphenicol (CHL).

70 μM, a range similar to that obtained for ciprofloxacin against wild-type gyrase.

Selection of resistant mutants. The frequency of selecting resistant mutants of *S. aureus* ATCC 29213 by ACH-702 at 4× MIC after 48 h of incubation at 37°C was approximately 9×10^{-11}, which was approximately 1 to 2 logs lower than that by ciprofloxacin (1×10^{-9} to 1.1×10^{-8}) at 4× MIC (not shown); this value for ciprofloxacin was in agreement with previously published values (38). For MRSA strains, the frequency of mutant selection by ACH-702 at 4× MIC was $<1 \times 10^{-10}$ for ATCC 33591 and $<4 \times 10^{-10}$ for BK2384. These frequencies were ≥2 logs lower than those observed (approximately 1×10^{-8}) using ciprofloxacin selection at 4× MIC for the two strains. Additional incubation of plates to 72 h yielded tiny ACH-702-resistant colonies at approximately 10-fold higher frequencies than those at 48 h, suggesting a fitness cost to these cells.

Macromolecular synthesis inhibition. From the above enzyme inhibition data, it would follow that ACH-702 is a selective inhibitor of DNA synthesis in bacteria. To verify this assumption, three macromolecular synthesis inhibition assays were performed with ACH-702 and control antibiotics to evaluate [³H]thymidine, [³H]uridine, and [³H]leucine incorporation into DNA, RNA, and protein, respectively, after a short exposure to the compounds. The results of these assays are summarized in Fig. 3. Three antibacterial agents with validated mechanisms of action were tested as controls. As expected, ACH-702 selectively inhibited DNA synthesis in a dose-dependent manner. The control antibiotic inhibitors, ciprofloxacin, rifampin, and chloramphenicol, showed the predicted inhibition patterns for DNA, RNA, and protein synthesis, respectively.

Therapeutic efficacy and pharmacokinetics in mice. Therapeutic efficacy was demonstrated in a mouse thigh infection model using immunocompromised mice. The *S. aureus* ATCC 33591 (MRSA) inoculum concentration was 2.2×10^6 CFU/ml with 0.1 ml injected per thigh. The mean density of bacteria in control thighs at the time of antibiotic treatment (2 h postinfection) was 8.17×10^4 CFU/ml. The number of untreated control CFU/ml increased over 2.7 logs by 24 h postinfection
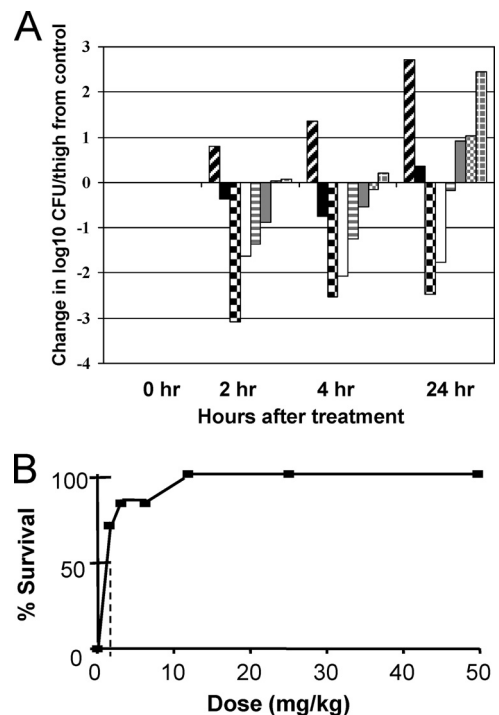

FIG. 4. *In vivo* efficacy in two mouse infection models. (A) Mouse thigh infection. ACH-702 and vancomycin were dosed by subcutaneous injection at time zero, which is 2 h postinfection, with MRSA strain ATCC 33591. The numbers of CFU per thigh were calculated at the indicated time points; the graph depicts the change at the indicated time compared to the untreated control at time zero. Symbols: diagonal lines, untreated controls; black filled, vancomycin (10 mg/kg); checkered, ACH-702 (20 mg/kg); white filled, ACH-702 (10 mg/kg); horizontal lines, ACH-702 (5 mg/kg); gray filled, ACH-702 (2.5 mg/kg); gray dotted, ACH-702 (1.25 mg/kg); gray crosshatch, ACH-702 (0.625 mg/kg). (B) Mouse sepsis. ACH-702 was dosed at the indicated dose levels by subcutaneous injection 1 h after infection with *S. aureus* ATCC 13709 (see Materials and Methods). The *y* axis indicates survival after 5 days. The dotted line indicates the approximate PD₅₀.

(Fig. 4A). A standard vancomycin subcutaneous treatment at 10 mg/kg resulted in reductions in bacterial populations of 0.37 and 0.74 and an increase of 0.36 logs at 2, 4, and 24 h after antibiotic treatment, respectively. Subcutaneous administration of ACH-702 displayed a dose-dependent effect on the MRSA infection with a 24-h static dose of approximately 5 mg/kg (decrease of 0.18 logs). A 2.47-log reduction of the number of CFU/ml was observed at 24 h at a dose level of 20 mg/kg. The two lowest dose levels, 0.625 and 1.25 mg/kg, resulted in only minimal decreases in the number of CFU/ml compared with untreated control numbers. Efficacy was also observed with a mouse sepsis model using immunocompetent mice and *S. aureus* ATCC 13709, with a 50% protective dose (PD₅₀) of approximately 0.5 mg/kg after subcutaneous administration of ACH-702, using a single dose at each concentration (Fig. 4B).

Pharmacokinetic parameters for ACH-702 in the plasma of treated mice were determined (Table 7). Mice were given a single subcutaneous dose at each concentration. At 10 mg/kg, the AUC from 0 to 24 h (AUC₀₋₂₄) for ACH-702 was 0.94 μg · h/ml, which increased to 5.73 μg · h/ml at 40 mg/kg. The

TABLE 7. Plasma pharmacokinetic parameters after subcutaneous administration to mice[a]

Drug	Dose (mg/kg)	C_{max} (µg/ml)	AUC_{0-24} (µg/ml)	$t_{1/2}$ (h)	t_{max} (h)
ACH-702	10	0.45	0.94	ND	1.0
	40	1.41	5.73	3.1	1.0
Ciprofloxacin	20	3.36	5.95	1.07	0.5
	50	7.42	14.43	1.17	0.5

[a] Definitions of pharmacokinetic parameters can be found in reference 25. ND, not determined.

maximum concentrations of drug in serum (C_{max}s) were 0.45 and 1.41 µg/ml, respectively, at the two dosing concentrations. These values were lower than the exposures seen for ciprofloxacin at 20 mg/kg, for which the AUC_{0-24} and C_{max} were 5.95 µg · h/ml and 3.36 µg/ml, respectively (32). This decreased exposure relative to that for ciprofloxacin was subsequently discovered to be the result of a more extensive glucuronidation of ACH-702 that reduced the levels of the parent compound (data not shown).

DISCUSSION

ACH-702, an isothiazoloquinolone (ITQ), represents an optimized analog of earlier compounds that were previously reported (9, 32, 43, 44, 45) to have reduced protein binding, enhanced inhibition of target enzymes, and improved *in vitro* and *in vivo* antibacterial activity. This compound displays potent *in vitro* antibacterial activity against both Gram-positive and Gram-negative bacteria (Tables 2 to 4), with particular effectiveness against resistant Gram-positive strains, including MRSA (Tables 2 and 5). The MICs against Gram-positive bacteria were generally superior to those of the comparators tested, including vancomycin, linezolid, and levofloxacin. The improved antibacterial activity of ACH-702 was particularly evident against fluoroquinolone-resistant isolates. Good activity (MICs of <1 µg/ml) was also observed against *Haemophilus*, *Moraxella*, and *Neisseria* strains as well as anaerobes, *Legionella*, and *Mycoplasma* clinical isolates, with the possible exception of *C. difficile*, for which the MIC_{90} was 4 µg/ml when tested against only 10 isolates. Activity against other Gram-negative bacteria was somewhat more limited but still was present for the majority of tested strains, as MIC_{50} values were ≤1 µg/ml (Table 3). Some improved antibacterial activity was seen against fluoroquinolone-resistant *Enterobacteriaceae* and *A. baumannii* compared with levofloxacin and ciprofloxacin. Good *in vitro* antibacterial activity was also demonstrated against *Mycobacterium* and *Nocardia* sp. (24, 34, 42).

A possible reason for the exceptional antibacterial activity of ACH-702 against Gram-positive bacteria is the potent dual targeting of both gyrase and topoisomerase IV enzymes, including those from quinolone-resistant strains, as demonstrated by the staphylococcal enzyme assays (Table 6). This is further supported by the observation that the effects of the quinolone-resistant phenotypes of the Gram-positive clinical isolates on the ACH-702 MIC_{90} values were not as significant as the major effects observed for quinolone MICs, which elevated many of them beyond their breakpoints. Also, laborato-

ry-selected quinolone-resistant mutants were found to retain susceptibility to ACH-702 (Table 5). The effective targeting of two bacterial enzymes simultaneously may also account for the slow selection of resistant mutants and could prolong the clinical effectiveness of a drug with such a profile (37). The reduced antibacterial activity of ACH-702 against some Gram-negative strains could be the result of reduced permeability, more effective efflux of the compound, or a combination of both. This has not been further explored at present. As might be expected for a compound with potent activity on two targets, the frequencies of selection of spontaneous resistant mutants are quite low. In fact, mutants were difficult to obtain under previously reported conditions used for quinolones at 4× MIC for 48 h at 37°C (38). We did observe a 10-fold or greater increase in selection frequency if plates were allowed to incubate for an additional 24 h or longer (total of ≥72 h). However, the colonies were tiny and grew much more slowly, indicating potential fitness costs to the bacteria. How this would translate to infection conditions in the host is unclear.

Similar to quinolones, ACH-702 was found to be rapidly bactericidal against representative Gram-positive (*S. aureus* and *E. faecalis*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria (Fig. 2), which may be a desirable property when treating a serious bacterial infection. At concentrations above 4× MIC, no further increases in the rates of killing were observed. Against the two Gram-positive strains, ACH-702 displayed greater bactericidal activity than both moxifloxacin and vancomycin, with greater killing evident at earlier times of exposure. Against the two Gram-negative strains, ACH-702 was comparable to ciprofloxacin and moxifloxacin in regard to both rate and extent of bacterial killing. Of note, a "rebound" effect was sometimes seen for the Gram-negative strains, especially with *P. aeruginosa*, where numbers of CFU/ml increased to nearly untreated control levels after 24 h. This rebound was observed for both ACH-702 and moxifloxacin against *P. aeruginosa*. The cause of this rebound effect is unknown. ACH-702 also demonstrated a postantibiotic effect (PAE) comparable to that of moxifloxacin in several *S. aureus* strains. This is another potential advantage during treatment of infections, as it could enhance the effective exposure of drug in a patient and contribute positively to successful clinical outcomes.

Therapeutic efficacy for ACH-702 was demonstrated in a mouse thigh infection model utilizing immunocompromised animals (Fig. 4A) and in a mouse sepsis model using immunocompetent animals (Fig. 4B). The 24-h static dose in the mouse thigh model against *S. aureus* was 5 mg/kg. The *in vivo* effectiveness of this compound is likely due to a combination of a low MIC for the infecting bacterial strains and relatively good pharmacokinetics in mice, although the plasma exposure was less than that of ciprofloxacin (Table 7). Efficacy was also observed with a mouse sepsis model with a PD_{50} value of 0.5 mg/kg and in a mouse lung infection model (*Streptococcus pneumoniae*) (M. Pucci, unpublished data). These studies were done with single subcutaneous doses, and improved results might be possible with multiple doses to compensate for the relatively short pharmacokinetic half-life in rodents. If exposure parameters track with ciprofloxacin, there is a reasonable expectation that half-lives and exposures will improve in other mammalian species, including humans. Preliminary data sug-

gest that the ratio of the AUC to the MIC appears to be the most important pharmacokinetic/pharmacodynamic (PK/PD) driver for ACH-702, as with fluoroquinolones (W. A. Craig, unpublished data). We believe that this compound has good tissue distribution, as exposure levels determined from thigh tissue were similar to plasma levels in mice. Clinical observations of all animals treated with ACH-702 in these studies were normal at the doses tested.

ACH-702 demonstrates excellent Gram-positive antibacterial activity, including against MRSA strains, using both *in vitro* and *in vivo* assays. In addition, unlike most current antibiotics used to treat MRSA infections, it possesses Gram-negative antibacterial activity. Future efforts might generate further optimized derivatives that display improved potency against these pathogens. These data along with impressive bactericidal activity and postantibiotic effects suggested that ACH-702 might work well in animal infection models. Indeed, the compound was effective in treating animal bacterial infections, as efficacy was observed with murine sepsis, lung, and thigh infection models. However, pharmacokinetic analyses revealed that there were significantly lower exposures in mice than with ciprofloxacin. This was found to be due to rapid metabolism of the parent compound via glucuronidation, adversely affecting intravenous and especially oral administration of this compound. Other nonsystemic indications for ACH-702 are under investigation. We are currently working to identify more metabolically stable derivatives, as we believe that the overall profile of isothiazoloquinolones and the potential to treat serious antibiotic-resistant pathogens warrant further study of this class.

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