In Vitro Antibacterial Activity of AZD0914, a New Spiropyrimidinetrione DNA Gyrase/Topoisomerase Inhibitor with Potent Activity against Gram-Positive, Fastidious Gram-Negative, and Atypical Bacteria

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AZD0914 is a new spiropyrimidinetrione bacterial DNA gyrase/topoisomerase inhibitor with potent *in vitro* antibacterial activity against key Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Streptococcus agalactiae*), fastidious Gram-negative (*Haemophilus influenzae* and *Neisseria gonorrhoeae*), atypical (*Legionella pneumophila*), and anaerobic (*Clostridium difficile*) bacterial species, including isolates with known resistance to fluoroquinolones. AZD0914 works via inhibition of DNA biosynthesis and accumulation of double-strand cleavages; this mechanism of inhibition differs from those of other marketed antibacterial compounds. AZD0914 stabilizes and arrests the cleaved covalent complex of gyrase with double-strand broken DNA under permissive conditions and thus blocks religation of the double-strand cleaved DNA to form fused circular DNA. Whereas this mechanism is similar to that seen with fluoroquinolones, it is mechanistically distinct. AZD0914 exhibited low frequencies of spontaneous resistance in *S. aureus*, and if mutants were obtained, the mutations mapped to *gyrB*. Additionally, no cross-resistance was observed for AZD0914 against recent bacterial clinical isolates demonstrating resistance to fluoroquinolones or other drug classes, including macrolides, β -lactams, glycopeptides, and oxazolidinones. AZD0914 was bactericidal in both minimum bactericidal concentration and *in vitro* time-kill studies. In *in vitro* checkerboard/synergy testing with 17 comparator antibacterials, only additivity/indifference was observed. The potent *in vitro* antibacterial activity (including activity against fluoroquinolone-resistant isolates), low frequency of resistance, lack of cross-resistance, and bactericidal activity of AZD0914 support its continued development.

With the continuing spread of methicillin-resistant *Staphylococcus aureus* (MRSA) in both community and hospital settings, increases in vancomycin resistance (1) and heteroresistance in *S. aureus* (2), outbreaks of linezolid resistance in staphylococci (3), and the recent emergence of ceftriaxone-resistant *Neisseria gonorrhoeae* in Japan (4, 5), new antibacterials, particularly those with an oral treatment option (6), are needed to treat infections caused by these Gram-positive and fastidious Gram-negative organisms.

AZD0914 is a new orally active spiropyrimidinetrione bacterial DNA gyrase/topoisomerase inhibitor with a novel mode of action that is being developed to help fill this need (7, 8, 9). The antibacterial activity of AZD0914 was shown to be via inhibition of DNA biosynthesis and accumulation of double-strand cleavages; this mechanism of action differs from those of other marketed antibacterial compounds, including fluoroquinolones. AZD0914 stabilizes and arrests the cleaved covalent complex of gyrase with double-strand broken DNA under permissive conditions and thus blocks religation of the double-strand cleaved DNA to form fused circular DNA (10, 11). Whereas this mechanism is similar to that seen with fluoroquinolones, it is mechanistically distinct. This novel mode of action of AZD0914 is further supported by the observation that fluoroquinolone- and methicillin-resistant staphylococci remain susceptible to AZD0914, as do ciprofloxacin-, ceftriaxone-, penicillin-, and tetracycline-resistant *N. gonorrhoeae* strains.

The goal of this study was to determine the *in vitro* spectrum of AZD0914 activity against a collection of Gram-positive, Gram-negative, atypical, and anaerobic bacterial isolates.

MATERIALS AND METHODS

Antibacterials. The structure of the compound AZD0914 is shown in Fig. 1. The antibacterial compounds included in this study were obtained from the following sources. AZD0914 and linezolid were from AstraZeneca Pharmaceuticals LP (Waltham, MA); ciprofloxacin was from MP Biomedicals (Santa Ana, CA); coumermycin A1 and metronidazole were from Sigma-Aldrich (St. Louis, MO); and aztreonam, amoxicillin, azithromycin, ceftazidime, ceftriaxone, cefixime, clavulanic acid, clindamycin, erythromycin, gentamicin, levofloxacin, meropenem, novobiocin, penicillin, rifampin, tetracycline, and vancomycin were from the United States Pharmacopeial Convention (Rockville, MD).

Bacterial isolates. The majority of the bacterial clinical isolates included in this study were from the AstraZeneca Research Collection (designated ARC) and were selected for susceptibility testing for their phenotypic or genotypic resistance characteristics. Additional isolates were

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FIG 1 Structure of AZD0914.

obtained from the North American *Staphylococcus aureus* Repository (BEI Resources, Manassas, VA [formerly NARSA]) or the American Type Culture Collection (ATCC), Manassas, VA, and also included Clinical and Laboratory Standards Institute (CLSI) quality control reference strains. Bacterial culture identifications were confirmed by standard microbiological methods (12) or through the use of an automated instrument (MicroScan WalkAway40 SI; Siemens Healthcare Diagnostics, Deerfield, IL).

Susceptibility testing. The MICs and minimum bactericidal concentrations (MBCs) obtained for each organism-drug combination were determined according to CLSI guidelines (13, 14, 15). The susceptibility interpretive criteria used for reference compounds along with the quality control ranges used for reference bacterial strains are described in CLSI documents M100-S24 (16) and M45-A2 (17). Whenever possible, the MIC range, MIC₅₀, and MIC₉₀ were reported for each species. Broth microdilution MICs for *Legionella pneumophila* were determined in *Legionella* broth (18). Isolates of *L. pneumophila* were initially grown on buffered charcoal yeast extract (Becton Dickinson, Sparks, MD) agar plates for 48 h prior to transfer and susceptibility testing. Anaerobic MICs were determined in a Bactron II anaerobe chamber (Sheldon Manufacturing Inc., Cornelius, OR) with a gas mixture containing 5% H₂, 5% CO₂, and 90% N₂.

MICs were determined by either CLSI broth microdilution methodology (with slight deviation) or agar dilution (*N. gonorrhoeae* and *Clostridium difficile*). For broth microdilution testing, stock compound plates were prepared and liquid handling automation (Tecan Freedom EVO or a Perkin-Elmer MiniTrak MultiPosition liquid handling robot) was utilized to spot 2- μ l aliquots of serial 2-fold drug dilutions into columns 1 to 11 of 96-well daughter plates. Column 12 contained no drug (solvent only) and served as the growth control. An inoculum volume of 98 μ l containing 5×10^5 CFU/ml in the appropriate broth medium was then inoculated directly into each well of the 96-well plate. Following appropriate incubation (all incubations were at 35°C; *N. gonorrhoeae* was incubated in 5% CO₂), the MICs were read visually and the MIC range, MIC₅₀, and MIC₉₀ were determined for organism groups containing ≥ 10 isolates. At least one CLSI quality control reference organism and control compound was used to validate susceptibility testing to ensure that there was no unacceptable variation between test dates for the control compounds.

MBC testing. MBC testing was performed by plating the 100- μ l well contents from the initial MIC testing (1 dilution below the MIC to ≥ 4 dilutions above the MIC) onto fresh Trypticase soy agar with 5% sheep blood plates (TSAw5%; Becton Dickinson) by the surface viable-count method (19). Compounds were considered bactericidal at the lowest drug concentration that reduced viable organism counts by $\geq 3 \log_{10}$ in 24 h. Inoculated plates were inverted and incubated overnight, viable colonies were counted, and MBCs were determined. Drug carryover was reduced by ≥ 250 -fold sample dilution into the agar plate.

In vitro time-kill studies. AZD0914 broth macrodilution MICs were determined and used as the starting point for both *in vitro* time-kill and postantibiotic-effect (PAE) tests. *In vitro* static time-kill studies were conducted with glass tubes (18 by 150 mm, without agitation) containing 10-ml volumes of cation-adjusted Mueller-Hinton broth (CAMHB; Becton Dickinson) with logarithmically growing cultures (starting inoculum of 1×10^6 CFU/ml) against levofloxacin-susceptible (USA300) and levo-

floxacin-resistant (USA100) *S. aureus*. AZD0914 was tested at concentrations equivalent to 0.5, 1, 2, 4, and 8 times the MIC; samples were plated for colony counts at 0, 2, 4, 6, 8, and 24 h by using 100- μ l aliquots spotted onto 25-ml sheep blood agar plates as described previously. Compounds were considered bactericidal at the lowest drug concentration that reduced viable organism counts by $\geq 3 \log_{10}$ in 24 h. Time-kill studies were conducted in duplicate; tests were combined, and mean values were reported.

PAE testing. PAE testing of AZD0914 and comparator compounds was conducted with glass tubes (18 by 150 mm) containing 10 ml CAMHB and logarithmically growing cultures of levofloxacin-susceptible (USA300) and levofloxacin-resistant (USA100) *S. aureus* at a starting inoculum of $\sim 1 \times 10^6$ CFU/ml. Cultures were exposed to AZD0914 concentrations equivalent to 0.5, 1, 4, or 16 times the MIC for 1 h; this was followed by removal of the compound by dilution (1:1,000) and monitoring for continued suppression of bacterial growth over time. The PAE was defined as the difference between the times required for the cultures in the compound-exposed and -unexposed tubes to increase by 1 $\log_{10}$ CFU/ml above the number present immediately after dilution of the compound.

Frequency-of-resistance determinations. The frequency of spontaneous *S. aureus* ARC1692 and USA300 resistance to AZD0914, levofloxacin, and linezolid was measured (in triplicate) by plating 10^9 to 10^{10} CFU/ml onto Mueller-Hinton agar (Becton Dickinson) plates containing 2, 4, and 8 times the agar dilution MIC. Inoculated plates were incubated for 48 h at 36°C in ambient atmosphere, and the colonies were counted. Plates were reincubated for an extended time and also checked at 96 h. Resistant colonies were confirmed by serial passage on drug-free medium; this was followed by broth microdilution susceptibility testing. The frequency of spontaneous resistance to AZD0914, levofloxacin, and linezolid was calculated as the ratio of the number of confirmed resistant colonies to the total number of viable cells plated from the starting inoculum. If no colonies were observed on the compound-containing agar plates, the frequency of spontaneous resistance development was expressed as less than the frequency at which one resistant colony was obtained.

Characterization of gyrase/topoisomerase mutants. The PCR primers used for *gyrA*, *gyrB*, *parC*, and *parE* are described below. PCR amplification and sequencing of the genes encoding the A subunits of DNA gyrase and topoisomerase IV subunit genes were carried out with chromosomal DNA from the *S. aureus* and *S. pneumoniae* isolates. Whole-genomic DNA was prepared from selected isolates with a standard genomic DNA preparation kit (Promega, Madison WI) and quantitated with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Fifty nanograms of genomic DNA was used as the template for amplification in a PCR with the Hi-Fidelity PCR System kit (F531S; Roche, Pleasanton, CA) and an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA). The cycling conditions were a 30-s denaturation at 94°C, followed by primer annealing for 30 s at 55°C and a 3-min extension at 72°C. The PCR product was purified with a QIAquick PCR Purification kit (Qiagen, Valencia, CA) and sequenced in an Applied Biosystems (Life Sciences) 3100 Series genetic analyzer (Thermo Fisher Scientific Inc., Waltham, MA). The oligonucleotide primers used for amplification of the *S. aureus* genes were as follows: *gyrA*, 5'-AAGCTTGAA GATGCGATTGAAGCGGACC-3' (forward) and 5'-CACCATCAAGAC TTATCAATGAAATACC-3' (reverse); *parC*, 5'-TGACAAAGTACAACC TAGACGTGAATGG-3' (forward) and 5'-AATTTAACGATAAGTACTT GGTCAGC-3' (reverse); *gyrB*, 5'-TAGATGATTCCGCGTCAAACG-3' (forward) and 5'-AGTATACGACGATGTACTGG-3' (reverse). *parE* was amplified in two portions with forward primer 5'-AACGAACGTACGTT TGCAGG-3', forward 1 primer 5'-CCTGAACGAGCATCTTCACG-3', reverse primer 5'-GTACGTACTAAAGATGGTGG-3', and reverse 1 primer 5'-TATTGAATTCAGTATTTCTCTCTCATC-3'.

The oligonucleotide primers used for amplification of the *S. pneumoniae* genes were as follows: *gyrA*, 5'-GTCACGAATATGCCTTATAGT TCTCG-3' (forward) and 5'-GCTAACTTGATGACTTATTGGTATTC C-3' (reverse); *parC*, 5'-TGGTTACTTATCGTAACTGAACACGGG-3'

TABLE 1 *In vitro* antibacterial activities of AZD0914 and comparators

Organism (no. of strains) and antibacterial agent	MIC ^f range	MIC ₅₀ ^f	MIC ₉₀ ^f
Gram-positive organisms			
<i>S. aureus</i> (100)			
AZD0914	0.06 to 0.5	0.25	0.25
Levofloxacin	0.125 to >16	8	>16
Linezolid	1 to >32	2	4
Vancomycin	0.5 to >32	1	8
MRSA (67)			
AZD0914	0.125 to 0.5	0.25	0.25
Levofloxacin	0.125 to >16	>16	>16
Linezolid	1 to >32	2	4
Vancomycin	0.5 to >32	1	>32
Levofloxacin-resistant <i>S. aureus</i> (54)			
AZD0914	0.125 to 0.5	0.25	0.25
Levofloxacin	4 to >16	>16	>16
Linezolid	1 to >32	2	4
Vancomycin	0.5 to >32	1	>32
VISA or VRSA (11)			
AZD0914	0.125 to 0.25	0.25	0.25
Levofloxacin	8 to >16	>16	>16
Linezolid	1 to 4	2	2
Linezolid-resistant <i>S. aureus</i> (3) ^a			
AZD0914	0.25 to 0.5		
Levofloxacin	16 to >16		
Linezolid	8 to >32		
Vancomycin	0.5 to 1		
<i>S. epidermidis</i> (39)			
AZD0914	0.06 to 0.25	0.25	0.25
Levofloxacin	0.125 to >16	0.25	16
Linezolid	0.125 to 32	1	2
Vancomycin	0.5 to 4	2	2
Levofloxacin-resistant <i>S. epidermidis</i> (16)			
AZD0914	0.125 to 0.25	0.25	0.25
Levofloxacin	4 to >16	8	16
Linezolid	0.125 to 32	1	2
Vancomycin	1 to 4	2	2
<i>S. haemolyticus</i> (21)			
AZD0914	0.125 to 1	0.125	0.25
Levofloxacin	0.125 to >16	0.125	>16
Linezolid	1 to 2	1	2
Vancomycin	0.25 to 2	1	2
<i>S. lugdunensis</i> (13)			
AZD0914	0.25 to 0.5	0.5	0.5
Levofloxacin	0.125 to 0.5	0.25	0.5
Linezolid	0.25 to 2	1	1
Vancomycin	0.5 to 1	1	1
<i>S. saprophyticus</i> (16)			
AZD0914	0.125 to 2	0.25	0.5
Levofloxacin	0.125 to 0.5	0.5	0.5
Linezolid	1 to 2	2	2
Vancomycin	0.5 to 4	1	2

TABLE 1 (Continued)

Organism (no. of strains) and antibacterial agent	MIC ^f range	MIC ₅₀ ^f	MIC ₉₀ ^f
<i>Staphylococcus</i> spp. (10) ^b			
AZD0914	0.06 to 0.5	0.25	0.5
Levofloxacin	0.125 to >16	0.25	0.5
Linezolid	1 to 2	2	2
Vancomycin	0.5 to 2	1	2
<i>S. pyogenes</i> (100)			
AZD0914	0.06 to 0.5	0.125	0.25
Levofloxacin	0.125 to 2	0.5	1
Erythromycin	≤0.015 to >16	0.06	2
Erythromycin-resistant <i>S. pyogenes</i> (13)			
AZD0914	0.06 to 0.25	0.125	0.25
Erythromycin	1 to >16	8	>16
<i>S. agalactiae</i> (100)			
AZD0914	0.06 to 0.25	0.125	0.25
Levofloxacin	0.5 to >16	1	1
Vancomycin	0.25 to 1	0.5	0.5
Erythromycin	0.03 to >16	0.06	>16
Erythromycin-resistant <i>S. agalactiae</i> (30)			
AZD0914	0.06 to 0.25	0.125	0.25
Erythromycin	2 to >16	16	>16
<i>S. pneumoniae</i> (98)			
AZD0914	0.06 to 0.25	0.125	0.25
Levofloxacin	0.25 to >16	1	16
Amoxicillin	≤0.015 to 8	0.06	1
Erythromycin	≤0.015 to >16	0.25	>16
Levofloxacin-resistant <i>S. pneumoniae</i> (18)			
AZD0914	0.06 to 0.25	0.125	0.25
Levofloxacin	8 to >16	16	>16
Amoxicillin	≤0.015 to 4	0.03	1
Erythromycin	≤0.015 to >16	2	>16
Erythromycin-resistant <i>S. pneumoniae</i> (46)			
AZD0914	0.06 to 0.5	0.125	0.25
Levofloxacin	0.5 to >16	1	16
Amoxicillin	≤0.015 to 8	0.5	4
Erythromycin	2 to >16	>16	>16
<i>E. faecalis</i> (51) ^c			
AZD0914	0.125 to 2	1	2
Levofloxacin	0.5 to >16	2	>16
Linezolid	1 to 32	2	16
Vancomycin	0.5 to >32	2	4
Levofloxacin-resistant <i>E. faecalis</i> (18)			
AZD0914	0.125 to 2	0.5	1
Levofloxacin	16 to >16	>16	>16
Linezolid	2 to >32	2	32
Vancomycin	1 to >32	1	>32
<i>E. faecium</i> (50) ^d			
AZD0914	0.25 to 16	8	16
Levofloxacin	0.5 to >16	>16	>16

(Continued on following page)

TABLE 1 (Continued)

Organism (no. of strains) and antibacterial agent	MIC ^f range	MIC ₅₀ ^f	MIC ₉₀ ^f
Linezolid	1 to >32	2	32
Vancomycin	0.5 to >32	16	>32
<i>L. monocytogenes</i> (10)			
AZD0914	0.125 to 1	0.5	1
Levofloxacin	0.5 to 1	1	1
Penicillin	0.125 to 0.5	0.25	0.25
Fastidious Gram-negative organisms			
<i>H. influenzae</i> (29)			
AZD0914	0.125 to 1	0.25	1
Levofloxacin	≤0.008 to 0.015	0.015	0.015
Ampicillin	0.06 to >16	0.5	>16
<i>N. gonorrhoeae</i> (37) ^c			
AZD0914	0.03 to 0.25	0.06	0.125
Ciprofloxacin	0.001 to >8	0.125	>8
Azithromycin	0.06 to 1	0.5	0.5
Ceftriaxone	0.002 to 2	0.015	0.03
Penicillin	0.008 to 32	0.5	32
Tetracycline	0.06 to 32	0.5	16
Ciprofloxacin-resistant <i>N. gonorrhoeae</i> (16)			
AZD0914	0.03 to 0.125	0.06	0.125
Ciprofloxacin	4 to >8	>8	>8
Azithromycin	0.06 to 1	0.5	1
Ceftriaxone	0.004 to 2	0.015	0.03
Penicillin	0.03 to 32	0.5	32
Tetracycline	0.06 to 32	0.5	32
Atypical organism			
<i>L. pneumophila</i> (10)			
AZD0914	0.008 to 0.06	0.03	0.06
Levofloxacin	0.008 to 0.015	0.015	0.015
Erythromycin	0.06 to 0.25	0.125	0.25
Gram-negative organisms			
<i>A. baumannii</i> (19)			
AZD0914	2 to >128	4	32
Meropenem	0.5 to >128	64	>128
Ceftazidime	4 to >128	>128	>128
<i>E. coli</i> (20)			
AZD0914	2 to 8	2	4
Levofloxacin	0.03 to >16	0.03	16
Ceftazidime	0.06 to >16	0.125	>16
<i>K. pneumoniae</i> (20)			
AZD0914	16 to >128	64	128
Meropenem	0.03 to 128	0.06	128
Ceftazidime	0.5 to >128	128	>128
<i>P. aeruginosa</i> (22)			
AZD0914	16 to >64	>64	>64
Levofloxacin	0.25 to 16	1	>16
Ceftazidime	0.25 to >16	2	>16

TABLE 1 (Continued)

Organism (no. of strains) and antibacterial agent	MIC ^f range	MIC ₅₀ ^f	MIC ₉₀ ^f
Anaerobes			
<i>Bacteroides</i> species (7)			
AZD0914	2 to 8		
Clindamycin	0.125 to >64		
Metronidazole	1 to 4		
<i>C. difficile</i> (2)			
AZD0914	0.125		
Clindamycin	1 to 4		
Metronidazole	0.125 to 0.25		

^a Includes two *cf* mutant isolates and one G2576T mutant isolate.

^b Includes two *Staphylococcus capitis*, one *S. sciuri*, one *S. simulans*, and six *S. warneri* isolates.

^c Includes seven linezolid-resistant and five vancomycin-resistant isolates.

^d Of the 50 isolates tested, 42 were levofloxacin resistant, 13 were linezolid resistant, and 24 were vancomycin resistant.

^e Of the 37 isolates tested, 3 were azithromycin resistant, 1 was ceftriaxone resistant, 16 were ciprofloxacin resistant, 9 were penicillin resistant, and 7 were tetracycline resistant.

^f Values are in micrograms per milliliter.

(forward) and 5'-TCAATCGTTACTGTCATATTCCACTCC-3' (reverse). The *gyrB* and *parE* genes were each amplified in two pieces. The oligonucleotide primers used for amplification were as follows: *gyrB*, forward primer 5'-CTCTGGTCTTGTCATTGGTGATCGGC-3', forward 1 primer 5'-ACCTGAAACCTTGATCCACCACC-3', reverse primer 5'-GAAGTGGTCAAGATTACCAATCGC-3', and reverse 1 primer 5'-TTA GACATCAAGTGACTATAGAC-3'; *parE*, forward primer 5'-ATACTC CTATTATATCATGAATTGGG-3', forward 1 primer 5'-ACGATCACC AAACCCAGACAAGGC-3', reverse primer 5'-TTCAGACTATCGTGAG GGACTAGC-3', and reverse 1 primer 5'-CTTTTTCACATAGTCATTC ACATCCG-3'.

Combination (checkerboard/synergy) testing. The antibacterial activity of AZD0914 was evaluated in combination with 17 antibacterial compounds by *in vitro* checkerboard/synergy testing with two-dimensional MIC arrays by the method of Lorian (20) against CLSI quality control reference strains, including *S. aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *S. pneumoniae* (ATCC 49619), *Escherichia coli* (ATCC 25922), and *Haemophilus influenzae* (ATCC 49247). The compounds tested in combination with AZD0914 included: aztreonam, amoxicillin, amoxicillin-clavulanic acid, azithromycin, ceftazidime, ceftriaxone, cefixime, clavulanic acid, clindamycin, gentamicin, levofloxacin, linezolid, meropenem, metronidazole, rifampin, tetracycline, and vancomycin. MICs of antibacterial agent combinations were measured as fold MIC reductions based on the original MIC of each of the compounds tested separately. Fractional inhibitory concentrations (FICs) were calculated as the MIC of

TABLE 2 MBCs of AZD0914 and comparators for *S. aureus* and *S. pyogenes*

Organism	Strain information	MIC/MBC μg/ml		
		AZD0914	Levofloxacin	Linezolid
<i>S. aureus</i>	ATCC 29213	0.125/0.25	0.125/0.25	4/>16
<i>S. aureus</i>	ATCC 25923	0.25/0.5	0.125/0.25	2/>16
<i>S. aureus</i>	ARC516, MSSA ^a	0.125/0.125	0.125/0.25	2/>16
<i>S. aureus</i>	ARC517, MRSA	0.25/0.5	4/4	1/>16
<i>S. aureus</i>	ATCC 33591, MRSA	0.125/0.125	0.125/0.25	2/>16
<i>S. aureus</i>	USA300, MRSA	0.25/0.25	0.5/1	2/>16
<i>S. aureus</i>	USA100, MRSA	0.125/0.25	8/16	4/>16
<i>S. pyogenes</i>	ARC838	0.125/0.125	0.5/1	1/1

^a MSSA, methicillin-susceptible *S. aureus*.

FIG 2 *In vitro* time-kill performance of AZD0914 (MIC, 0.25 µg/ml) against levofloxacin-resistant MRSA (USA100).

drug A tested in combination divided by the MIC of drug A alone plus the MIC of drug B tested in combination divided by the MIC of drug B alone. The mean FIC index (FICI) was obtained by adding all of the individual FICs and dividing by the number of data points. The mean FICs were interpreted as synergistic when values were ≤ 0.5 , additive/indifferent when values were > 0.5 to 4.0 , and antagonistic when values were > 4 . When the MIC of a compound for a reference strain was greater than the highest concentration tested, the next highest MIC was used for FIC calculations. Similarly, when a compound's MIC for a reference strain was less than the lowest concentration tested, the lowest MIC tested was used for the FIC calculation.

RESULTS

The antibacterial spectrum of AZD0914 and comparators evaluated in MIC₉₀ studies against a collection of 774 Gram-positive, Gram-negative, atypical, and anaerobic bacterial clinical isolates and isolates enhanced for resistance is presented in Table 1. AZD0914 was the most active compound tested against Gram-positive organisms, including *S. aureus* (MRSA, vancomycin-intermediate *S. aureus* [VISA], and vancomycin-resistant *S. aureus* [VRSA]), *S. epidermidis*, and streptococci (*Streptococcus agalactiae*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*), with a MIC₉₀ of 0.25 µg/ml. In these organism groups, AZD0914 was equally active against isolates resistant to erythromycin, levofloxacin, linezolid, and/or vancomycin. No cross-resistance to AZD0914 was observed in the fluoroquinolone-resistant isolates. Compared to the staphylococci and streptococci, somewhat less AZD0914 activity against *Listeria monocytogenes* and *Enterococcus faecalis* was observed (MIC₉₀s of 1 and 2 µg/ml, respectively) whereas the AZD0914 MIC₉₀ was notably higher for *E. faecium* (16 µg/ml).

AZD0914 was very active against fastidious Gram-negative organisms and atypical organisms, including *H. influenzae*, *L. pneumophila*, and *N. gonorrhoeae*. The MIC ranges for these groups

FIG 3 *In vitro* time-kill performance of AZD0914 (MIC, 0.25 µg/ml) against *S. pneumoniae* ATCC 49619.

FIG 4 PAE of AZD0914 (MIC, 0.25 µg/ml) against levofloxacin-resistant MRSA (USA100).

were 0.125 to 1, 0.008 to 0.06, and 0.03 to 0.25 µg/ml, respectively. The corresponding MIC₉₀s were 1, 0.06, and 0.125 µg/ml. No cross-resistance to AZD0914 was observed in 16 *N. gonorrhoeae* isolates (MIC range, 0.03 to 0.125 µg/ml; MIC₉₀, 0.125 µg/ml) resistant to ciprofloxacin (MIC range, 4 to > 8 µg/ml; MIC₉₀, > 8 µg/ml). AZD0914 was also very active (MIC, 0.06 µg/ml) against a recent clinical isolate of *N. gonorrhoeae* (5) resistant to both ceftriaxone and ciprofloxacin (MICs, 2 and ≥ 16 µg/ml, respectively).

AZD0914 was less active against Gram-negative organisms, including *Acinetobacter baumannii*, *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, with MIC₉₀s of 32, 4, 128, and > 64 µg/ml, respectively. The MICs for efflux pump mutants of *E. coli* ATCC 25922 (*tolC*) and *P. aeruginosa* PAO1 (*mexABCDXY*) were ≥ 32 -fold lower than those for the corresponding parent strains, indicating that efflux plays a role in MICs for Gram-negative organisms.

In a small study of anaerobic pathogens, AZD0914 was highly active against *C. difficile* (MIC, 0.125 µg/ml) and less active against *Bacteroides* spp. (MICs, 2 to 8 µg/ml).

In MBC testing against seven *S. aureus* strains and one *S. pyogenes* strain, AZD0914 was bactericidal against all of the isolates tested (including fluoroquinolone-resistant isolates), with MBCs within a 2-fold range of the MIC (Table 2). The bactericidal activity of AZD0914 against *S. aureus* USA100 (levofloxacin resistant) and *S. pneumoniae* ATCC 49619 was confirmed by *in vitro* time-kill studies (Fig. 2 and 3), where AZD0914 produced a ≥ 3 -log reduction in the viable-organism counts of both strains within 6 h at concentrations ≥ 2 times the MIC.

The PAE of AZD0914 was also evaluated against *S. aureus* USA100 (levofloxacin resistant) and *S. pneumoniae* ATCC 49619

FIG 5 PAE of AZD0914 (MIC, 0.25 µg/ml) against *S. pneumoniae* ATCC 49619.

TABLE 3 Frequencies of spontaneous resistance to AZD0914, levofloxacin, and linezolid in *S. aureus*

Isolate and fold MIC	Frequency of resistance at 48 h		
	AZD0914	Levofloxacin	Linezolid
ARC1692 (MRSA)			
2	1.9×10^{-9}	TNTC ^a	$<7.8 \times 10^{-10}$
4	$<6.3 \times 10^{-10}$	TNTC	$<7.8 \times 10^{-10}$
8	$<6.3 \times 10^{-10}$	5.2×10^{-7}	$<7.8 \times 10^{-10}$
USA300 (MRSA)			
2	3.8×10^{-9}	1.7×10^{-7}	TNTC
4	$<2.2 \times 10^{-10}$	4.2×10^{-9}	$<1.0 \times 10^{-9}$
8	$<2.2 \times 10^{-10}$	1.0×10^{-9}	$<1.0 \times 10^{-9}$

^a TNTC, too numerous to count.

(Fig. 4 and 5). The PAE values of AZD0914 ranged from 0.25 to 0.55 h at the MIC, from 1.65 to 2.4 h at 4 times the MIC, and from 2.15 to >3.3 h at 16 times the MIC. The observed PAE values of AZD0914 are similar to results previously reported for quinolones and fluoroquinolones against *S. aureus* at 10 times the MICs (21, 22).

The frequency of spontaneous development of resistance to AZD0914 in *S. aureus* was very low and comparable to that of linezolid (Table 3). The AZD0914 resistance frequencies ranged from 1.9×10^{-9} to 3.8×10^{-9} at 2 times the MIC to $<2.2 \times 10^{-10}$ at 4 and 8 times the MIC. In comparison, levofloxacin-resistant mutants were obtained at all of the concentrations tested, whereas only the AZD0914 concentration equal to 2 times the MIC resulted in resistant colonies in *S. aureus* ARC1692 and none of USA300 were obtained. The AZD0914-resistant colonies were analyzed, and if the MIC increased ≥ 4 -fold, the mutations mapped to *gyrB*.

The antibacterial activities of AZD0914 and comparators against individual isolates of *S. aureus* and *S. pneumoniae* with characterized gyrase and topoisomerase mutations were evaluated (Table 4). A no-more-than-2-fold variation in the AZD0914 MIC was observed between isolates lacking gyrase/topoisomerase mutations and clinical isolates possessing frequently encountered mutations in *gyrA*, *gyrB*, *parC*, or *parE* known to cause resistance

to fluoroquinolones (23, 24), novobiocin (25), or coumermycin (26), indicating a general lack of cross-resistance to AZD0914 due to these common gyrase and topoisomerase mutations.

The antibacterial activity of AZD0914 was assessed in combination with 17 comparator antibacterials in checkerboard/synergy studies with five CLSI quality control reference strains. On the basis of mean FICI values, no synergy or antagonism was detected with any of the combinations tested. All checkerboard combination mean FICI values were considered either additive or indifferent. The lowest mean FICI observed (0.77) was for AZD0914 combined with gentamicin against *E. coli*, and the highest mean FICI was 1.66 for AZD0914 combined with aztreonam against *H. influenzae*. The MICs of the comparator compounds (aztreonam, amoxicillin, amoxicillin-clavulanic acid, azithromycin, ceftazidime, ceftriaxone, cefixime, clavulanic acid, clindamycin, gentamicin, levofloxacin, linezolid, meropenem, metronidazole, rifampin, tetracycline, and vancomycin) were within the CLSI quality control ranges for each of the reference strains tested.

DISCUSSION

In 2013, the Centers for Disease Control and Prevention issued a report describing antibiotic resistance threats in the United States citing drug-resistant *N. gonorrhoeae* as an urgent health threat; MRSA and drug-resistant *S. pneumoniae* as serious health threats; and VRSA, erythromycin-resistant group A streptococci, and clindamycin-resistant group B streptococci as concerning health threats (27). In response to this medical need, we report on the *in vitro* antibacterial activity of a new orally active spiropyrimidinetrione bacterial DNA gyrase/topoisomerase inhibitor (AZD0914) possessing a novel mechanism of action distinct from that of fluoroquinolones, with potent *in vitro* antibacterial activity against key Gram-positive and fastidious Gram-negative pathogens, including susceptible and drug-resistant isolates of *S. aureus*, *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, and *N. gonorrhoeae*, including isolates with known resistance to fluoroquinolones and ceftriaxone. Importantly, no cross-resistance to AZD0914 was observed in bacterial clinical isolates expressing resistance to other drug classes, including macrolides (azithromycin and erythromycin), β -lactams (amoxicillin, ampicillin, ceftazidime, ceftriaxone, meropenem,

TABLE 4 *In vitro* antibacterial activities of AZD0914 and comparators against *S. aureus* and *S. pneumoniae* isolates with defined DNA gyrase/topoisomerase mutations

Strain	Gyrase/topoisomerase mutation(s)	MIC (μ g/ml)			
		AZD0914	Levofloxacin	Novobiocin	Coumermycin A1
<i>S. aureus</i> ARC516	None	0.125	0.125	0.125	≤ 0.015
<i>S. aureus</i> ARC2379	<i>gyrA</i> P80T, S84L, A457T; <i>parC</i> S80F, V693M; <i>gyrB</i> K65Q; <i>parE</i> N141S	0.25	16	0.25	≤ 0.015
<i>S. aureus</i> ARC2375	<i>gyrA</i> S84L, A457T; <i>parC</i> S80Y, E84G, V693M; <i>gyrB</i> K65Q; <i>parE</i> N141S	0.25	>16	0.25	≤ 0.015
<i>S. aureus</i> ARC2380	<i>gyrA</i> S84L, E88K, A457T; <i>parC</i> S80Y, E84G, P135S, Y410F, V693M; <i>gyrB</i> K65Q; <i>parE</i> N141S	0.25	>16	0.06	≤ 0.015
<i>S. aureus</i> ARC2790	<i>gyrB</i> T173A	0.125	0.125	0.5	0.03
<i>S. aureus</i> ARC3445	<i>gyrB</i> R144I	0.125	0.25	16	2
<i>S. pneumoniae</i> NCTC 7446	None	0.125	0.5	0.5	0.125
<i>S. pneumoniae</i> ARC2481	<i>gyrA</i> E85K; <i>parC</i> S79Y, K137N; <i>gyrB</i> I163V, K169R, T177I, I188T	0.25	>16	0.5	0.06
<i>S. pneumoniae</i> ARC2801	<i>gyrA</i> D81N	0.25	1	0.5	0.125
<i>S. pneumoniae</i> ARC3035	<i>gyrB</i> T172A	0.25	0.5	8	8

and penicillin), glycopeptides (vancomycin), lincosamides (clindamycin), or oxazolidinones (linezolid).

The *in vitro* spectrum of AZD0914 also encompassed atypical and anaerobic bacterial pathogens, including *L. pneumophila* (MIC₉₀, 0.06 µg/ml) and *C. difficile* (MICs, 0.125 µg/ml), as well as pathogens frequently encountered in sexually transmitted infections often coinfecting patients with *N. gonorrhoeae*, including *Chlamydia trachomatis* and *Mycoplasma genitalium* (28, 29).

AZD0914 was comparable to fluoroquinolones in exhibiting bactericidal activity (confirmed by MBC and *in vitro* time-kill studies), a short PAE, and general additivity/indifference in *in vitro* checkerboard/synergy testing. However, it is noteworthy that AZD0914 exhibited a low frequency of resistance development in *S. aureus* (if MICs increased, the mutations mapped to *gyrB*), lacked cross-resistance in clinical isolates expressing common quinolone/fluoroquinolone resistance mutations, including staphylococci (*gyrA* S84L and *parC* S80F), streptococci (*gyrA* E85K and *parC* S79Y), and *N. gonorrhoeae* (*gyrA* S91F and D95G), and remained active against isolates resistant to novobiocin or coumermycin, confirming the novel mechanism of action of this compound.

The potent *in vitro* antibacterial activity of AZD0914 against key Gram-positive and fastidious Gram-negative bacterial species with an urgent or serious unmet medical need (including activity against fluoroquinolone-resistant isolates), a low frequency of resistance, a lack of cross-resistance to other drug classes, and bactericidal activity, supports the continued development of AZD0914 and this chemical series. In addition, AZD0914 has recently received a qualified infectious disease product designation and fast-track status and is currently being evaluated for the treatment of sexually transmitted infections caused by *N. gonorrhoeae*.

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