Inhibition of Neisseria gonorrhoeae Type II Topoisomerases by the Novel Spiropyrimidinetrione AZD0914* 

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Background: Inhibition of Neisseria gonorrhoeae type II topoisomerases gyrase and TopoIV by the antibacterial spiropyrimidinetrione AZD0914 was investigated.

Results: AZD0914 stabilized the gyrase-DNA complex with double strand DNA cleavage, retaining potency in a fluoroquinolone-resistant mutant, with little inhibition of human type II topoisomerases.

Conclusion: AZD0914 displays mechanistic differences from fluoroquinolones.

Significance: AZD0914 has the potential to combat drug-resistant gonorrhea.

We characterized the inhibition of Neisseria gonorrhoeae type II topoisomerases gyrase and topoisomerase IV by AZD0914 (AZD0914 will be henceforth known as ETX0914 (Entasis Therapeutics)), a novel spiropyrimidinetrione antibacterial compound that is currently in clinical trials for treatment of drug-resistant gonorrhea. AZD0914 has potent bactericidal activity against N. gonorrhoeae, including multidrug-resistant strains and key Gram-positive, fastidious Gram-negative, atypical, and anaerobic bacterial species (Huband, M. D., Bradford, P. A., Otterson, L. G., Basrab, G. S., Giacobe, R. A., Patey, S. A., Kutschke, A. C., Johnstone, M. R., Potter, M. E., Miller, P. F., and Mueller, J. P. (2014) In Vitro Antimicrobial Activity of AZD0914: A New Spiropyrimidinetrione DNA Gyrase/Topoisomerase Inhibitor with Potent Activity against Gram-positive, Fastidious Gram-negative, and Atypical Bacteria. Antimicrob. Agents Chemother. 59, 467–474). AZD0914 inhibited DNA biosynthesis preferentially to other macromolecules in Escherichia coli and induced the SOS response to DNA damage in E. coli. AZD0914 stabilized the enzyme-DNA cleaved complex for N. gonorrhoeae gyrase and topoisomerase IV. The potency of AZD0914 for inhibition of supercoiling and the stabilization of cleaved complex by N. gonorrhoeae gyrase increased in a fluoroquinolone-resistant mutant enzyme. When a mutation, conferring mild resistance to AZD0914, was present in the fluoroquinolone-resistant mutant, the potency of ciprofloxacin for inhibition of supercoiling and stabilization of cleaved complex was increased greater than 20-fold. In contrast to ciprofloxacin, relocation of the cleaved DNA did not occur in the presence of AZD0914 upon removal of magnesium from the DNA-gyrase-inhibitor complex. AZD0914 had relatively low potency for inhibition of human type II topoisomerases α and β.

In 2013 the United States Centers for Disease Control and Prevention classified the threat level associated with the unmet medical need resulting from multidrug-resistant Neisseria gonorrhoeae as urgent (1), and it estimated that at least 800,000 cases of gonorrhoeae occur per year in the United States alone (2).

Fluoroquinolone antibacterial drugs previously offered an effective treatment option for gonorrhea. Over the last decade, however, development of resistance, first against fluoroquinolones and subsequently against all drugs used for first line treatment, such as cefixime and ceftriaxone (3, 4), demanded the development of novel agents to combat highly resistant N. gonorrhoeae.

Fluoroquinolones, one of the most successful classes of antibiotics on the market (5, 6), target the homologous bacterial type II topoisomerases gyrase and topoisomerase IV (TopoIV).2 Both enzymes are conserved across most bacterial pathogens and are essential for cellular functions, including DNA replication and decatenation. DNA gyrase, a heterotetramer of two subunits, GyrA2-GyrB2, introduces negative supercoils in DNA ahead of the replication fork, thereby relieving torsional strain during replication (6–8). TopoIV, a ParC2-ParE2 heterotetramer, catalyzes decatenation, which is essential for separating linked catenanes of two DNA molecules during replication.

Type II topoisomerases modulate the topology of DNA in eukaryotes (6–11). The human nuclear type II topoisomerases TopoIα and -β are the targets of inhibitors that have clinical utility for the treatment of cancer (12, 13). Sufficient selectivity by antibacterial drugs for inhibition of the bacterial over human topoisomerases at clinically relevant doses has been achieved, encouraging continued exploration of these enzymes as viable targets for novel antibacterial drugs.

The molecular mechanism of type II topoisomerases is described by a functional model termed the two-gate mechanism (14–16). The catalytic cycle has several stages that can be

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2 The abbreviations used are: TopoIV, topoisomerase IV; TopoII, topoisomerase II; CC50, potency for stabilization of cleaved complex; MIC, minimum inhibitory concentration; TEV, tobacco etch virus; IPTG, isopropyl 1-thio-β-D-galactopyranoside.
Aldrich. Human TopoII

cell wall, triclosan blocked labeled acetic acid incorporation into fatty acids, and the aminocoumarin novobiocin as well as the fluoroquinolone norfloxacin blocked DNA synthesis.

**SOS Induction Assay**

The SOS induction assay was performed as described (23).

**DNA Manipulations and Plasmid Construction**

For wild type *N. gonorrhoeae* gyrase, the following plasmids were used: pJT1330 (*N. gonorrhoeae* GyrA-TEV-His$_6$), pJT1331 (His$_6$-TEV-*N. gonorrhoeae* ParE), pJT1337 (His$_6$-TEV-*N. gonorrhoeae* GyrB), pETite N-His (T7-based expression plasmid), and pETite C-His KAN (T7-based expression plasmid). The primers used were as follows: NgogyrARev (5'-GACATCGGAGATATACATGACAGCAACCATGCCGCCCCACC-3'), NgogyrBRev (5'-CATATGGACATCACCCCACTACGTCGCGCCGAACACCTGTTTTCAAGGATCCTCCATCTGAACAAAAACACGAAG-3'), NgogyrBRev (5'-GACATCGGAGATATACATGACAGCAACCATGCCGCCCCACC-3'), NgogyrBRev (5'-GACATCGGAGATATACATGACAGCAACCATGCCGCCCCACC-3'), and NgoparERev (5'-GACATCGGAGATATACATGACAGCAACCATGCCGCCCCACC-3').

**Inhibition of Macromolecule Biosynthesis**

The procedure was performed according to Hilliard (10), with modifications published previously (22). *Escherichia coli* was grown at room temperature in cation-adjusted Mueller Hinton Broth 1 (Sigma-Aldrich) in the presence of radiolabeled precursors. As positive controls, rifamycin blocked the incorporation of labeled uridine into RNA, erythromycin blocked the incorporation of labeled valine and leucine incorporation into protein, penicillin G blocked labeled N-acetylglucosamine incorporation into the

**Experimental Procedures**

**Materials**

Buffers, salts, and routine biochemicals were sourced from Sigma-Aldrich and were of reagent grade or higher purity. Plasmid NTC0109711-U6-shRNA, a derivative of pCR4-TOPO, was used in supercoiling, cleaved complex, and religation assays. Relaxation of the supercoiled form was done as described previously (20). It was obtained in supercoiled form from Nature Technologies (Lincoln, NE). Kinetoplast DNA used in decatenation assays was obtained from Topogen, Inc. (Port Orange, FL). Ciprofloxacin HCl was from MP Biomedicals (Santa Ana, CA). Etoposide and ATP were from Sigma-Aldrich. Human TopoII$\alpha$ was from Affymetrix (Santa Clara, CA). Human TopoII$\beta$ was supplied by Prof. Caroline A. Austin (University of Newcastle-upon-Tyne).

**Chemistry**

AZD0914 was characterized as described by Basarab et al. (21).

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pETite N-His or pETite C-His KAN vector. DNA sequences of cloned genes were confirmed by sequencing on an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA) using the Big Dye Terminator cycle sequencing kit (Applied Biosystems). Computer analysis of DNA sequences was performed with Sequencher (Gene Codes Corp., Ann Arbor, MI).

Plasmids for expressing either a TEV protease-cleavable N-terminal His<sub>6</sub>-tagged <i>N. gonorrhoeae</i> GyrB (pJT1337) or ParE (pJT1331) were made by amplifying the gene using the primers NgogyrBFor and NgogyrBRev (<i>gyrB</i>) or NgoparEFor and NgoparERev (<i>parE</i>) from genomic DNA by PCR. The PCR product was purified and cloned into the plasmid pETite N-His by ligation-independent cloning. The cloning reaction was transformed into Hi-Control 10G competent cells (Lucigen). Transformants were selected on LB + 25 μg/ml kanamycin, plasmids were isolated, and inserts were verified by PCR and sequencing.

Plasmid pJT1330 for expressing a TEV protease-cleavable C-terminal His<sub>6</sub>-tagged <i>N. gonorrhoeae</i> GyrA was constructed by amplifying the gene encoding <i>N. gonorrhoeae</i> GyrA from genomic DNA by PCR using the primers NgogyrAFor and NgogyrARev. The resulting PCR product was spin column-purified and cloned into the plasmid pETite C-His by ligation-independent cloning. The cloning reaction was transformed into Hi-Control 10G competent cells. Transformants were selected on LB + 25 μg/ml kanamycin, plasmids were isolated, and inserts were verified by PCR and sequencing.

Plasmid pJT1337 was used as a template for site-directed mutagenesis to create D429N and K450T mutations in separate point mutations was used for transformation. Plasmid pJT1337 was inoculated into LB medium with 25 μg/ml kanamycin at <i>A</i><sub>600</sub> = 0.1, incubated at 37 °C for 2 h, induced with 0.5 mM IPTG at <i>A</i><sub>600</sub> = 0.5, incubated for 3 h at 37 °C, harvested at <i>A</i><sub>600</sub> = 1.76 by centrifugation, and frozen at −20 °C. The purification followed the same protocol as described for GyrA. The yield of GyrB was 8.8 mg from 1 liter of cell paste. The protein was stored at −80 °C. To obtain GyrB protein carrying point mutations, the same procedure as described for wild type protein was used, except that the plasmid carrying the respective point mutations was used for transformation.

<i>N. gonorrhoeae</i> ParE—To produce ParE protein, <i>E. coli</i> Hi-Contro BL21(DE3) cells transformed with plasmid pJT1337 were inoculated into LB medium with 25 μg/ml kanamycin at <i>A</i><sub>600</sub> = 0.1, incubated at 37 °C for 3.5 h, induced with 0.5 mM IPTG at <i>A</i><sub>600</sub> = 0.6, incubated for 3.5 h at 30 °C, harvested at <i>A</i><sub>600</sub> = 1.7 by centrifugation, and frozen at −20 °C. Cell paste from 2 liters of culture expressing ParE was extracted and subjected to Ni<sup>2+</sup>-chelating chromatography as for GyrA, except that the flow-through fractions containing ParE were pooled and concentrated to 5 ml by an Amicon<sup>®</sup> Ultracel-10K concentrator (Millipore, Billerica, MA) using XbaI and Xhol restriction sites to create plasmid pNG055.

To produce ParC protein, <i>E. coli</i> BL21(DE3) cells transformed with plasmid pNG055 were inoculated into LB medium with 25 μg/ml kanamycin at <i>A</i><sub>600</sub> = 0.1, incubated at 30 °C for 3.5 h, induced with 0.5 mM IPTG at <i>A</i><sub>600</sub> = 0.6, incubated for 3.5 h at 30 °C, harvested at <i>A</i><sub>600</sub> = 1.7 by centrifugation, and frozen at −20 °C. The purification followed the same protocol as described for GyrA. The yield was 25 mg from 1 liter of cell paste.

<i>N. gonorrhoeae</i> GyrB—To produce protein GyrB, <i>E. coli</i> Hi-Contro BL21(DE3) cells transformed with plasmid pJT1337 were inoculated into LB medium with 25 μg/ml kanamycin at <i>A</i><sub>600</sub> = 0.1, incubated at 37 °C for 2 h, induced with 0.5 mM IPTG at <i>A</i><sub>600</sub> = 0.5, incubated for 3 h at 37 °C, harvested at <i>A</i><sub>600</sub> = 1.76 by centrifugation, and frozen at −20 °C. The purification followed the same protocol as described for GyrA. The yield was 25 mg from 1 liter of cell paste. To obtain GyrB protein carrying point mutations, the same procedure as described for wild type protein was used, except that the plasmid carrying the respective point mutations was used for transformation.

<i>N. gonorrhoeae</i> GyrA—To produce GyrA protein, <i>E. coli</i> Hi-Control BL21(DE3) cells transformed with plasmid pJT1337 were inoculated into LB medium with 25 μg/ml kanamycin at <i>A</i><sub>600</sub> = 0.1, incubated at 37 °C for 3 h, induced with 0.5 mM IPTG at <i>A</i><sub>600</sub> = 0.5, incubated overnight at 37 °C, harvested at <i>A</i><sub>600</sub> = 1.76 by centrifugation, and frozen at −20 °C. The purification followed the same protocol as described for GyrA. The yield was 25 mg from 1 liter of cell paste. To obtain GyrA protein carrying point mutations, the same procedure as described for wild type protein was used, except that the plasmid carrying the respective point mutations was used for transformation.

<i>N. gonorrhoeae</i> ParE—To produce ParE protein, <i>E. coli</i> Rosetta (DE3) cells transformed with plasmid pJT1331 were inoculated into LB medium with 25 μg/ml kanamycin at <i>A</i><sub>600</sub> = 0.1, incubated at 30 °C for 3.5 h, induced with 0.5 mM IPTG at <i>A</i><sub>600</sub> = 0.6, incubated for 3 h at 30 °C, harvested at <i>A</i><sub>600</sub> = 1.7 by centrifugation, and frozen at −20 °C. The purification followed the same protocol as described for GyrA. The yield of ParE was 21 mg from 1 liter of cell paste.
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**TABLE 1**

| Gyrase reconstitution conditions | Ratio (A:B) | SC | CC |
|---------------------------------|------------|----|----|
| **Gyrase** | **GyrB** | **Ratio (A:B)** | **SC** | **CC** |
| WT | WT | 1:2 | 2 | 20* |
| WT | WT | 1:8 | 2 | 40 |
| WT | D429N | 1:4 | 5 | 50 |
| WT | K450T | 1:8 | 10 | 50 |
| S91F,D95G | WT | 1:2 | 20 | 50 |
| S91F,D95G | K450T | 2:3 | 20 | 50 |

* Religation assay used the same conditions.

Ciprofloxacin and AZD0914 were dissolved in dimethyl sulfoxide (DMSO), which contributed 1% of the final volume for all assays. Because mutant enzymes displayed differences in specific activity of up to 10-fold, the concentrations of individual protein subunits were adjusted accordingly. To arrive at final assay conditions, each subunit was varied with the other in limiting quantity. Reconstitutions to form active enzyme were performed as described previously (20). Final enzyme concentrations are described such that the limiting subunit concentration is presumed to define one-half of the reconstituted enzyme. These concentrations and the ratio of subunits used in assays are given in Table 1. The composition of TopoIV used in the decatenation and cleaved complex assays were 2 and 20 nM, respectively, with a 1:2 ratio of ParC/ParE.

**Enzyme Assays**

All assays were conducted at room temperature, ~21 °C. Ciprofloxacin and AZD0914 were dissolved in dimethyl sulfoxide (DMSO), which contributed 1% of the final volume for all assays. Because mutant enzymes displayed differences in specific activity of up to 10-fold, the concentrations of individual protein subunits were adjusted accordingly. To arrive at final assay conditions, each subunit was varied with the other in limiting quantity. Reconstitutions to form active enzyme were performed as described previously (20). Final enzyme concentrations are described such that the limiting subunit concentration is presumed to define one-half of the reconstituted enzyme. These concentrations and the ratio of subunits used in assays are given in Table 1. The composition of TopoIV used in the decatenation and cleaved complex assays were 2 and 20 nM, respectively, with a 1:2 ratio of ParC/ParE.

**Gel Analysis and Quantitation**

All assays were evaluated by gel electrophoresis. Gel dimensions were 12 cm wide × 13.5 cm long × 0.7 cm thick. Samples (25 μl) were loaded into wells of 1% agarose gels buffered with 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA at pH 8.4 and run for 18–20 h at 30 V or 3 h at 60 V. For all gel assays except decatenation, 1 μg/ml ethidium bromide was included in the agarose gel running buffer. DNA bands for decatenation assays were visualized by staining with ethidium bromide (5 μg/ml in TAE (Tris base, acetic acid, and EDTA)) after electrophoresis. The DNA was quantified by AlphaEase software (Genetic Technologies) using digital images acquired during UV transillumination. For supercoiling and decatenation reactions, percentage of inhibition was determined directly from band intensities of supercoiled and unlinked DNA bands, referenced to control reactions containing no inhibitor. For cleaved complex and religation assays, background was subtracted based on control wells without inhibitor.

DNA supercoiling assays were carried out using a modification of the method of Mizuuchi (25). Assays were conducted in a 30-μl volume in buffer composed of 35 mM Tris-HCl (pH 7.5), 1.8 mM spermidine, 8 mM MgCl2, 24 mM KCl, 6.5% (w/v) glycerol, 0.005% Brij-35, 2 mM dithiothreitol, 400 ng of relaxed DNA plasmid, and 1 mM ATP. Reactions were initiated by the addition of enzyme and quenched by the addition of 6 μl of 0.5 M EDTA after 30 min, followed by 4 μl of DNA loading dye consisting of 40% sucrose, 100 mM Tris-HCl (pH 7.5), 1 mM EDTA-NaOH (pH 8), and 0.5 mg/ml bromphenol blue. Negative control wells were treated with the quench solution prior to initiation with enzyme.

DNA decatenation assays were performed in 30 μl at room temperature and contained 20 mM Tris-HCl (pH 8.0), 50 mM ammonium acetate, 5 mM dithiothreitol, 8 mM MgCl2, 0.5 mM EDTA, 5% (w/v) glycerol, 0.005% (w/v) Brij-35, 200 ng of kinetoplast DNA, and 1 mM ATP. Reactions were initiated with 2 nM TopoIV and allowed to react for 60 min prior to quenching and processing as described for DNA supercoiling. Negative controls were performed as for supercoiling assays.

For supercoiling and decatenation reactions, the amounts of product present in the negative and positive control reactions were used to define DNA band intensities for 100% (MIN) and 0% (MAX) inhibition, respectively. The percentage of inhibition at each inhibitor concentration was calculated with Equation 1,

\[
\% \text{ inhibition} = 100(1 - \frac{(x - \text{MIN})}{\text{MAX} - \text{MIN}})
\]

where \( x \) is the band intensity for the particular reaction. The half-maximal inhibitory concentration (IC\text{50}) was obtained by non-linear least squares regression of the percentage of inhibition data with Equation 2,

\[
\% \text{ inhibition} = 100\left(1 - \left(\frac{[I]}{IC_{50} + [I]}\right)^n\right)
\]

where \( n \) is the Hill coefficient, and \([I]\) is the inhibitor concentration.

Cleaved complex assays for gyrase and TopoIV were conducted similarly to the method described previously (26) with minor modifications to make conditions consistent with the supercoiling and decatenation assays described above. Reactions were performed in 30 μl under the same conditions used for supercoiling or decatenation, respectively, with the exception that enzyme concentrations were increased, as listed in Table 1. TopoIV assays used 400 ng of relaxed plasmid in place of kinetoplast DNA. Reaction times were 30 and 60 min for gyrase and TopoIV, respectively, after which 6 μl of 1% SDS containing 0.5 mg/ml proteinase K was added as a quench. Solutions were gently mixed and incubated at 37 °C for 30 min. DNA loading dye (4 μl) was added, and samples of 30 μl were analyzed by gel electrophoresis.

Assays probing religation of gyrase-cleaved complex used a modification of a published method (27). Assays were conducted in 30 μl with ciprofloxacin and AZD0914 included at 40 μM (>20-fold excess over cleaved complex IC\text{50} values). The cleaved complex was first formed in a 20-μl volume with all reaction components as described for the gyrase-cleaved complex assay, but at a 1.5-fold higher concentration to account for a subsequent dilution, and incubated 30 min. Religation was then induced by the addition of 10 μl of EDTA and sodium sulfate solutions, to variably buffer the free Mg\text{2+} concentration and balance the ionic strength at ~0.53 M for all conditions. Religation reactions were incubated for 45 min, quenched by the addition of 3 μl of 2% SDS and 1 mg/ml proteinase K, and incubated for 30 min at 37 °C. DNA loading dye (4 μl) was added to each reaction. DNA products were quantified by gel

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electrophoresis. The concentrations of EDTA and sodium sulfate in the final 30-μl volume were as follows: 104 and 0 mM, 81 and 42 mM, 58 and 83 mM, 35 and 125 mM, 23 and 146 mM, 12 and 166 mM, 6 and 176 mM, and 3 and 177 mM.

The concentration of free Mg$^{2+}$ for each condition in the religation assays was calculated from the indicated concentrations of total Mg$^{2+}$ (8 mM; $[M]_0$ and EDTA ($[L]_0$) using Equation 3.

$$[M]_{\text{free}} = [M]_0 - 1/2([M]_0 + [L]_0 + K_D)$$

$$- \sqrt{([M]_0 + [L]_0 + K_D)^2 - 4[M]_0[L]_0}$$

(Eq. 3)

The value of $K_D$, representing the pH-adjusted apparent affinity of EDTA for Mg$^{2+}$, was taken as 2.5 μM (28). Two protonation constants for EDTA and the Mg$^{2+}$-chelate stability constant (log $K_D$) were 10.19, 6.13, and 8.96, respectively (29). Ionic strength contributions to the buffer made by Mg$^{2+}$ speciation were determined for each solution by calculating the affinity of EDTA for Mg$^{2+}$ using Equation 4.

$$[L]_{\text{free}} = [L]_0 - ([M]_0 - [M]_{\text{free}})$$

(Eq. 4)

At pH 7.5, the protonation equilibrium of unliganded EDTA is 0.01:0.945:0.045 for the tetra-, tri-, and dianionic species (28). Finally, the excess ionic strength ($I$) contributed by EDTA for each condition was calculated with Equation 5.

$$I = 1/2(0.01 \times 16 \times [L]_{\text{free}} + 0.945 \times 9 \times [L]_{\text{free}} + 0.045 \times 4 \times [L]_{\text{free}})$$

(Eq. 5)

**Human Topoisomerase Supercoiling Assay**

The potency of compounds for inhibiting human TopoIα and -β, expressed as IC$_{50}$, was determined with a fluorescence anisotropy-based assay as described (30, 31).

**Results**

**Mechanism of Action of AZD0914**—The effect of AZD0914 on macromolecular biosynthesis pathways was investigated by tracking the incorporation of radioactively labeled precursors into actively growing E. coli cells (22). AZD0914 inhibited DNA biosynthesis with an IC$_{50}$ of 0.037 μg/ml for [3H]thymidine incorporation. The IC$_{50}$ for RNA biosynthesis was 4.3 μg/ml. The IC$_{50}$ values for protein, cell wall, and fatty acid biosynthesis were ≥256 μg/ml. This result is consistent with inhibition of DNA biosynthesis being the primary mechanism of action of AZD0914, as expected for a topoisomerase inhibitor.

**SOS Induction in E. coli**—When E. coli senses DNA damage or interference with DNA replication, a range of physiological changes known as the SOS response occurs (32–34). Induction of the E. coli SOS response was monitored using an E. coli ΔtolC biosensor strain containing a transcriptional fusion between the recA promoter and GFP on a low copy plasmid. Fig. 2 depicts the SOS response induced after exposure to the aminocoumarin novobiocin, the fluoroquinolone ciprofloxacin, and AZD0914. Novobiocin, a slow killing antibiotic that interferes with DNA gyrase by competing with ATP binding, induced a relatively low level, concentration-dependent SOS response. Ciprofloxacin, a rapidly bactericidal fluoroquinolone antibiotic, induced a high level SOS response. AZD0914, which is also rapidly bactericidal (35), induced a high level response similar to that observed for ciprofloxacin at concentrations below its MIC.

**Supercoiling and Decatenation**—AZD0914 and ciprofloxacin were compared with respect to their inhibitory potencies toward N. gonorrhoeae gyrase-catalyzed supercoiling and N. gonorrhoeae TopoIV-catalyzed decatenation (Fig. 3). Ciprofloxacin was slightly more potent than AZD0914 for gyrase inhibition (1.1 versus 6.3 μM IC$_{50}$, respectively) and TopoIV inhibition (6.3 versus 19 μM IC$_{50}$, respectively). For both compounds, inhibition of gyrase was more potent than inhibition of TopoIV.

**Cleaved Complex Assay**—Both ciprofloxacin and AZD0914 stabilized double strand-broken DNA bound to gyrase and TopoIV (cleaved complex). Whereas the compound concentrations required to produce 50% cleaved complex were similar for both compounds with gyrase (1.9 and 1.7 μM IC$_{50}$, respectively), ciprofloxacin was more potent than AZD0914 with TopoIV (0.065 versus 0.5 μM IC$_{50}$, respectively) (Fig. 4).

**Religation of Double Strand DNA in the Cleaved Complex**—Double strand-cleaved DNA in the DNA-N. gonorrhoeae gyrase complex stabilized by ciprofloxacin can be religated if Mg$^{2+}$ is removed from the complex (36). It has been proposed that this effect is due to the requirement for Mg$^{2+}$ in the enzyme-fluoroquinolone complex formation (37, 38). In this experiment, the stabilized cleaved complex between DNA gyrase, inhibitor, and DNA is preformed by preincubation with the respective inhibitor, ciprofloxacin or AZD0914, as described above. Subsequently, EDTA additions to the preformed cleaved complex were chosen to deplete the free Mg$^{2+}$ to concentrations between 0.1 μM and 1.7 mM. As expected, the cleaved complex formed in the presence of ciprofloxacin religated over a range of free Mg$^{2+}$ concentrations between 0.1 and
Effect of Resistance Mutations on Gyrase Inhibition by Ciprofloxacin and AZD0914—We prepared the N. gonorrhoeae GyrA subunit having both of two well characterized point mutations (S91F, D95G) in the quinolone resistance-determining region conferring resistance to fluoroquinolones (40–42), as well as separate N. gonorrhoeae GyrB subunits with one of two point mutations (K450T or D429N) from strains resistant to AZD0914 (39). These mutant subunits were reconstituted with either the wild type or mutated form of the other gyrase subunit. The effects of ciprofloxacin and AZD0914 on supercoiling and cleaved complex stabilization by the mutant enzymes were compared with wild type DNA gyrase (Table 2). All mutant forms had to be reconstituted at higher mutant subunit stoichiometries, as indicated in Table 1, to achieve comparable activities.

For gyrase with GyrA mutations conferring resistance to ciprofloxacin, the potencies of ciprofloxacin for inhibition of supercoiling (IC_{50}) and stabilization of the cleaved complex (CC_{50}) were more than 20-fold decreased (>500 for mutant versus 1–2 μM for wild type). AZD0914, however, showed a slightly enhanced inhibition of supercoiling (6 μM for wild type versus 2 μM for the GyrA mutant) and cleaved complex stabilization (1.7 μM for wild type versus 1 μM for the GyrA mutant).

Point mutations in the GyrB subunit conferring resistance to AZD0914 did not change the potency of ciprofloxacin with respect to supercoiling inhibition. Whereas the D429N mutation had no significant effect on the potency of cleaved complex stabilization by ciprofloxacin, the K450T mutation increased the potency by 6-fold. The D429N mutation had no effect on the supercoiling IC_{50} of AZD0914, and the K450T mutation elevated it only 2-fold. In contrast, both mutations substantially elevated the CC_{50} of AZD0914.

Surprisingly, when the K450T mutant GyrB subunit was reconstituted with the mutant GyrA subunit, the potencies for supercoiling inhibition and cleaved complex stabilization by ciprofloxacin were enhanced by more than 20-fold from >500 μM with the GyrA-only mutant to 24 μM for supercoiling and 19 μM for cleaved complex stabilization. In contrast, this enhancement of potency was not seen for AZD0914.

Selectivity for Bacterial Topoisomerases—Compared with its inhibition of N. gonorrhoeae gyrase, AZD0914 was a relatively weak inhibitor of the ATP-dependent supercoiled DNA relaxation activity of both human topoisomerase IIα and IIβ (IC_{50} = >400 and 79 μM, respectively). These potencies are comparable with those of ciprofloxacin (IC_{50} = 110 and 111 μM, respectively). In comparison, the anticancer drug etoposide, which targets human TopoII, showed more potent inhibition for both isozymes (IC_{50} = 11 and 13 μM, respectively) (Table 3).

Discussion

AZD0914 preferentially inhibits DNA biosynthesis over biosynthesis of other key macromolecules in sensitive bacteria. This is consistent with inhibition of type II topoisomerases by this compound. The large SOS response caused by AZD0914
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(Fig. 2) was similar to that seen with the fluoroquinolone ciprofloxacin, consistent with the observation that AZD0914, like ciprofloxacin, is a type II topoisomerase poison (i.e., it causes double strand DNA breaks due to cleaved complex stabilization). Inhibition of gyrase or topoisomerase IV by an ATP-competitive inhibitor, such as novobiocin, which does not cause direct DNA damage, resulted in a much smaller SOS response.

In *N. gonorrhoeae*, the rise of resistance to fluoroquinolones makes it imperative to identify new drugs, like AZD0914, that are able to maintain activity against resistant strains. Fluoroquinolone resistance resulting from mutations in the target type II topoisomerases has been attributed to a common mechanism, namely reduced affinity of inhibitor binding because of disruption of a “water-metal ion bridge” between the compound and GyrA or ParC (43, 44). It has been proposed that by minimizing the requirement for this bridge for binding affinity, as seen with quinazolinediones, compounds binding in the same region of these enzymes could overcome this resistance mechanism (45). Although there is as yet no x-ray structural confirmation of the binding site of AZD0914 or related spiropyrimidinetriones, based on mapping resistance mutations in GyrB, Alm and co-workers (39) proposed that AZD0914 occupies the same pocket in gyrase as fluoroquinolones. However, they propose that AZD0914 does not engage the non-catalytic magnesium ion coordinated through GyrA but rather interacts differently, through residues in GyrB that do not involve a chelating interaction with Mg^{2+}. The lack of a water-metal ion bridge to GyrA in the binding of AZD0914 with gyrase, therefore, is a potential explanation for the ability of this compound to maintain activity in fluoroquinolone-resistant strains.

One important feature to define in a new class of inhibitors that act as topoisomerase poisons is selectivity over human type II topoisomerases (Table 2). AZD0914 exhibited little inhibition of human TopoIIα (IC_{50} > 400 μM versus 110 μM for ciprofloxacin) and weak inhibition of human TopoIIβ (IC_{50} = 79 μM). The latter IC_{50} was comparable with that of ciprofloxacin.
FIGURE 5. Effect of magnesium depletion on the religation reaction of cleaved DNA-gyrase or DNA-TopoIV complex formed in the presence of AZD0914 or ciprofloxacin, respectively. A, example of religation reaction of DNA bound to gyrase inhibited by AZD0914 or ciprofloxacin, respectively. Final concentrations of free Mg$^{2+}$ are indicated above each well. Wells a and b were used as controls: a, no enzyme; b, enzyme, no compound. The plots below the gel images show nicked (N), linearized (L), and closed circular (C) DNA as a function of free Mg$^{2+}$ concentration. AZD0914 blocks religation, and ciprofloxacin does not. B, example of religation reaction of DNA bound to TopoIV inhibited by AZD0914 or ciprofloxacin, respectively. Final concentrations of free Mg$^{2+}$ are indicated above each well. Wells a and b were used as controls: a, no enzyme; b, enzyme, no compound. The plots to the right show nicked, linearized, and closed circular DNA at increasing amounts of MgCl$_2$. Religation occurs both in the presence of AZD0914 and ciprofloxacin.

TABLE 2
Effects of ciprofloxacin and AZD0914 on supercoiling and cleaved complex stabilization by mutant enzymes compared with wild type DNA gyrase

| Gyrase          | Supercoiling $IC_{50}$ | Cleavage complex $CC_{50}$ | MIC         |
|-----------------|------------------------|-----------------------------|-------------|
|                 |                        | CIP (μM) | AZD0914 (μM) | CIP (μM) | AZD0914 (μM) | CIP (μg/ml) | AZD0914 (μg/ml) |
| GyrA (WT)       | GyrB (WT)              | 1.1 ± 0.2 | 6.3 ± 0.2 | 1.9 ± 0.7 | 1.7 ± 0.3 | 0.008* | 0.125 |
| GyrA (S91F,D95G) | GyrB (WT)              | >500     | 2.0 ± 0.2 | >500     | 1 ± 0.4  | 16    | 0.125 |
| GyrA (WT)       | GyrB (K450T)           | 1 ± 0.3  | 13 ± 3   | 0.3 ± 0.01 | 6 ± 1   | 0.001 | 1    |
| GyrA (WT)       | GyrB (K429N)           | 1 ± 0.3  | 6 ± 1    | 3 ± 0.3  | 12 ± 0.4 | ND    | ND   |
| GyrA (S91F,D95G) | GyrB (K450T)           | 24 ± 0.4 | 19 ± 5   | 19 ± 1   | 12 ± 3  | 0.25  | 1    |

a MIC values are taken from Alm (43). The MIC value shown for GyrA (S91F,D95G) GyrB (K450T) is from a strain possessing GyrA (S91F,D95A) GyrB (K450T).

b ND, not determined.
(111 μM), which is well tolerated even after long term clinical administration (46). Thus, it is expected that AZD0914 should not exhibit significant mechanism-based toxicity in humans. Indeed, AZD0914 showed no in vitro mammalian genotoxicity and no measurable toxicity in an in vivo rat micronucleus assay (21).

A comparison of the MIC value of AZD0914 for N. gonorrhoeae (0.25 μg/ml = 0.5 μM) (4) with the observed IC50 and CC50 values for gyrase and TopoIV (IC50 = 6.3 and 19 μM, respectively, and CC50 = 1.7 and 0.5 μM, respectively (Figs. 3 and 4) reveals that the CC50 values are of a similar magnitude as the MIC90 value. This is a similar finding as with fluoroquinolones in E. coli and is explained by the acutely toxic nature of the DNA lesions created by fluoroquinolone action. However, in E. coli, fluoroquinolones display a potency asymmetry, with gyrase inhibited at lower concentrations than TopoIV (47). First-step mutants to fluoroquinolones in E. coli appear exclusively in gyrase and, in combination with the observed lower CC50 values for gyrase, serve to implicate gyrase as the primary fluoroquinolone target in E. coli. In N. gonorrhoeae, mutational studies also implicate gyrase as the primary fluoroquinolone target (41, 48), but surprisingly, in our enzyme assays, the CC50 values for ciprofloxacin were asymmetrical, with the TopoIV CC50 being 29-fold more potent than the CC50 for gyrase. This observation suggests that N. gonorrhoeae may be less susceptible to poisoning of TopoIV as opposed to gyrase. In this regard, the observation with fluoroquinolones in E. coli that TopoIV-mediated cell killing is slower than gyrase-mediated killing (49) may be relevant.

With AZD0914, first step and second step resistance mutations appear in GyrB, implicating gyrase as the primary target, despite a balanced biochemical profile between gyrase and TopoIV as measured in IC50 and CC50 assays, consistent with the hypothesis that poisoning of TopoIV in N. gonorrhoeae may not be as lethal as poisoning of gyrase. This hypothesis could be corroborated with future experiments employing strains engineered with single GyrB and ParE mutations or further with a recent technique that quantifies the amount of inhibitor-bound topoisomerase complexes in cells (50).

Both ciprofloxacin and AZD0914 stabilized the cleaved complex, but to differentiate between the mode of inhibition of AZD0914 and ciprofloxacin, we examined the potential of each drug to interfere with religation of double strand-nicked DNA bound to the topoisomerase tetramer. Investigators working on Staphylococcus aureus and E. coli topoisomerases demonstrated that ciprofloxacin inhibits the religation reaction and that Mg2+ stabilizes the interaction of fluoroquinolones with DNA (27, 36, 51). This interaction is maintained in the DNA topoisomerase-fluoroquinolone complex via the 3-carboxyl group on the fluoroquinolone and an aspartate/glutamate and a serine residue in helix IV of the GyrA subunit (38, 52). To compare ciprofloxacin and AZD0914, we first accumulated cleaved complex and then removed Mg2+ by the addition of EDTA. For the ciprofloxacin-induced DNA-gyrase cleaved complex, religation of the cleaved complex was observed at free Mg2+ concentrations between 0.2 and 100 μM. In contrast, very little religation was observed for the complex induced by AZD0914 at all concentrations of Mg2+ tested. This indicates that Mg2+ is not critically involved in the binding of AZD0914 to N. gonorrhoeae DNA gyrase and is a clear differentiation between the binding modes of AZD0914 and ciprofloxacin (Fig. 5A). This differentiation is further supported by the observation that there is no cross-resistance between ciprofloxacin-resistant N. gonorrhoeae strains and AZD0914 (35, 39). No religation occurred below 0.2 μM Mg2+, probably due to the removal of the Mg2+ from the catalytic site on DNA gyrase. This binding site must have a tighter affinity, and its occupation by Mg2+ may be required for the ligation reaction.

When the same experiment was conducted with TopoIV from N. gonorrhoeae, the cleaved complexes stabilized by both AZD0914 and ciprofloxacin religated when Mg2+ was removed (Fig. 5B). This is in contrast to the observation that the cleaved complex formed with E. coli or S. aureus TopoIV in the presence of AZD0914 cannot religate (data not shown). The result for the N. gonorrhoeae TopoIV enzyme is therefore unusual and may reflect a different mode of binding to TopoIV that involves Mg2+.

The AZD0914 mode of inhibition was further differentiated from that of ciprofloxacin by comparing the ability of both compounds to interact with wild type gyrase as well as previously described mutant versions of gyrase that confer resistance to either ciprofloxacin or AZD0914 (39) (Table 3). We studied the catalytic and inhibition properties of gyrase enzymes containing resistance mutations at loci in GyrA (Ser-91 and Asp-95) that disrupt the fluoroquinolone water-metal ion bridge and two loci for AZD0914 resistance in GyrB (35, 39). In the reconstituted fluoroquinolone-resistant gyrase, ciprofloxacin IC50 and concentrations where 50% cleaved complex is observed (CC50) were increased >250-fold from the wild type enzyme, whereas AZD0914 IC50 and CC50 values were unchanged. This result biochemically confirms why AZD0914 maintains activity in fluoroquinolone-resistant strains. The reconstituted gyrase with AZD0914-resistant mutations in GyrB displayed no differences in IC50 values to AZD0914 compared with wild type but did display 3–6-fold elevation in CC50 which agrees with the moderate MIC elevation (~8-fold) conferred by the Gy rB D292N and K450T mutations. Interestingly, the AZD0914-resistant gyrase containing the K450T Gy rB mutation displayed a 6-fold lowered CC50 for ciprofloxacin, which is reflected in the 8-fold reduced MIC that Gy rB K450T confers in a wild type background. Similarly, the gyrase tetramer carrying resistance mutations against both ciprofloxacin and AZD0914 (GyrA S91F D95G and Gy rB K450T) also displayed a lower CC50 for ciprofloxacin compared with the Gy rA tetramer that carried just the ciprofloxacin resistance mutations S91F and D95G. These biochemical findings corrobor-
orate that the K450T mutation, while conferring resistance to AZD0914, also conveys enhanced susceptibility to ciprofloxacin. They also further reinforce that although ciprofloxacin and AZD0914 both target DNA gyrase, they employ binding modes that are clearly distinct from each other. The observation that resistance against binding of AZD0914 sensitizes binding of ciprofloxacin to gyrase raises the question of whether concomitant dosing of ciprofloxacin and AZD0914 could prevent the emergence of resistant N. gonorrhoeae strains.

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