Probing the Differential Interactions of Quinazolinodione PD 0305970 and Quinolones with Gyrase and Topoisomerase IV^{▽†}

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Quinazoline-2,4-diones, such as PD 0305970, are new DNA gyrase and topoisomerase IV (topo IV) inhibitors with potent activity against gram-positive pathogens, including quinolone-resistant isolates. The mechanistic basis of dione activity vis-à-vis quinolones is not understood. We present evidence for *Streptococcus pneumoniae* gyrase and topo IV that PD 0305970 and quinolones interact differently with the enzyme breakage-reunion and Toprim domains, DNA, and Mg²⁺—four components that are juxtaposed in the topoisomerase cleavage complex to effect DNA scission. First, PD 0305970 targets primarily gyrase in *Streptococcus pneumoniae*. However, unlike quinolones, which select predominantly for *gyrA* (or topo IV *parC*) mutations in the breakage-reunion domain, unusually the dione selected for novel mutants with alterations that map to a region of the Toprim domain of GyrB (R456H and E474A or E474D) or ParE (D435H and E475A). This “dione resistance-determining region” overlaps the GyrB quinolone resistance-determining region and the region that binds essential Mg²⁺ ions, each function involving conserved EGDSA and PLRGK motifs. Second, dione-resistant gyrase and topo IV were inhibited by ciprofloxacin, whereas quinolone-resistant enzymes (GyrA S81F and ParC S79F) remained susceptible to PD 0305970. Third, dione-promoted DNA cleavage by gyrase occurred at a distinct repertoire of sites, implying that structural differences with quinolones are sensed at the DNA level. Fourth, unlike the situation with quinolones, the Mg²⁺ chelator EDTA did not reverse dione-induced gyrase cleavage nor did the dione promote Mg²⁺-dependent DNA unwinding. It appears that PD 0305970 interacts uniquely to stabilize the cleavage complex of gyrase/topo IV perhaps via an altered orientation directed by the bidentate 3-amino-2,4-dione moiety.

The established and emerging resistance of gram-positive pathogens to fluoroquinolones, macrolides, and beta-lactams has underlined the need for new antimicrobial agents (2, 6, 28, 32, 35, 46, 52). Recent work has shown that quinazolinodiones constitute a promising new class of antibacterial inhibitors of DNA gyrase and topoisomerase IV (topo IV) that share structural similarity with fluoroquinolones (14, 50, 51). Among this group of inhibitors, the 3-aminoquinazolinodione PD 0305970 (3-amino-7-[(R)-3-[(S)-1-aminoethyl]-pyrrolidin-1-yl]-1-cyclopropyl-6-fluoro-8-methyl-1H-quinazoline-2,4-dione) (Fig. 1) has particularly potent activity against susceptible and drug-resistant gram-positive species, especially *Streptococcus pneumoniae* (23). The drug differs most notably from quinolones, e.g., ciprofloxacin, in having the 3-aminoquinazoline 2,4-dione system in place of the 3-carboxy-substituted 4-quinolone ring (Fig. 1). Surprisingly, these subtle differences in drug structure confer activity against quinolone-resistant strains (23).

Initial work with *Escherichia coli* gyrase has shown that, like quinolones, 3-aminodiones, such as PD 0305970, stabilize an enzyme “cleavage complex” on DNA (50). This complex is thought to reflect the trapping of an intermediate in the topoisomerase reaction cycle in which the gyrase GyrA₂GyrB₂ (or

the topo IV ParC₂ParE₂) complex promotes the ATP-dependent passage of one DNA duplex through a transient double-strand break in a second DNA segment (8, 30). DNA scission in the cleavage complex is Mg²⁺-dependent and involves the covalent phosphotyrosyl linkage of the two gyrase GyrA subunits (ParC in topo IV) to the 5' phosphate ends of an enzyme-bridged double-stranded DNA break (5, 7–9, 16). Until recently, there was no high-resolution structure of a gyrase or topo IV cleavage complex, although information was available for some individual domains (3, 7–9, 22, 24, 31, 47, 55) and for yeast topo II (4, 12, 15, 29). By analogy with the yeast enzyme, it was reasoned that the N-terminal breakage-reunion domain of GyrA (ParC) (carrying the catalytic tyrosine) and the C-terminal Toprim metal binding domain of the GyrB (ParE) subunit come together to mediate DNA cleavage on each strand (7–9, 12, 13, 18, 34). Indeed, we have recently shown that the *S. pneumoniae* ParC breakage-reunion and ParE Toprim domains are sufficient to form a cleavage complex stabilized by moxifloxacin and clinafloxacin (25). Moreover, we have solved the crystal structures of these complexes, revealing two quinolone drugs intercalated at the highly bent DNA gate (25). It is known that different quinolones can preferentially target gyrase or topo IV in *Streptococcus pneumoniae* (1, 17, 20, 33, 38, 41), and these structures provide new insight into the nature of the cleavage complex.

Biochemical evidence points to the participation of two Mg²⁺ ions in DNA strand scission (11, 34, 49, 54). One Mg²⁺ ion is suggested to bind the 3' bridging oxygen of the scissile DNA phosphodiester bond, thereby promoting DNA cleavage by stabilizing the leaving 3'-OH group (11, 34). The function of the putative second ion is unclear but may involve potentiation

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FIG. 1. Structures of PD 0305970 and ciprofloxacin.

of the catalytic tyrosyl OH of GyrA (ParC), facilitating nucleophilic attack on the DNA phosphate (11, 34). Quinolones chelate free Mg^{2+} , but it is presently unclear if the drugs interact similarly with enzyme-bound Mg^{2+} (reviewed in reference 27). Quinolone resistance mutations occur in the so-called “quinolone resistance-determining regions” (QRDRs) located in the GyrA/ParC breakage reunion domain (10, 36, 57) and in the GyrB (ParE) Toprim fold (58).

To date, efforts with 3-hydroxy- and 3-amino-quinazolinodiones have established structure-activity relationships in vivo and inhibition of *E. coli* DNA gyrase in vitro (14, 19, 23, 50, 51). A preliminary report has identified three mutations in PD 0305970-resistant pneumococci that map in GyrB and ParE (23). However, little is known about how 3-aminodiones stabilize the topoisomerase cleavage complex vis-à-vis quinolones and how they overcome resistance. To initiate studies of this area, we have used a variety of genetic approaches in concert with functional enzyme and biochemical assays to examine the interaction of PD 0305970 with its topoisomerase targets in *S. pneumoniae*.

MATERIALS AND METHODS

Media and reagents. Preparation of Luria broth, Luria agar, and brain heart infusion medium containing 10% blood was as described previously (38–42). Sparfloxacin hydrochloride and ciprofloxacin hydrochloride were obtained from Daiinippon Pharmaceutical Co., Suita, Japan, and from Bayer AG, Wuppertal, Germany. Quinazolinodione PD0305970 and gemifloxacin were obtained from Pfizer, Ann Arbor, MI, and GlaxoSmithKline, Harlow, United Kingdom, respectively.

Bacterial strains and plasmids. *S. pneumoniae* 7785 and D5 have been described previously (38). The laboratory strain, *S. pneumoniae* R6, was from our strain collection, as were *E. coli* DH5 α used for cloning purposes and *E. coli* BL21(λ DE3) used for protein overexpression. The panel of isogenic 7785 mutants, 1C1, 2C6, 2C7, 1S1, 1S4, 2S1, and 3C4, has been described previously (42). Plasmids pXP9, pXP10, pXP13, and pXP14 used to express *S. pneumoniae* GyrB, GyrA, ParC, and ParE proteins and expression plasmids for GyrA S81F and ParC S79F have been described (43, 44). Supercoiled pBR322 and relaxed pBR322 were from New England BioLabs and John Innes Enterprises, Ltd. Kinetoplast DNA was purchased from TopoGEN.

Drug susceptibilities. Bacterial susceptibility to drugs was determined by a twofold dilution assay in which approximately 10^4 CFU of the *S. pneumoniae* strain was spotted onto brain heart infusion agar plates, which were assessed after overnight aerobic incubation at 37°C. The MIC is the drug concentration at which no growth was seen when tested under these conditions.

Stepwise selection for mutants. Mutants resistant to quinazolinone PD0305970 were obtained by stepwise challenge of *S. pneumoniae* strain 7785 by use of the procedure described previously for ciprofloxacin and other quinolones (41, 42). Briefly, approximately 5×10^9 CFU of strain 7785 was plated on brain heart infusion agar plates containing 10% horse blood and various concentrations of the dione. Resistant mutants appeared on plates containing drug at 0.03 μ g/ml (i.e., $1 \times$ MIC) after a 48-h aerobic incubation at 37°C (no colonies were recovered using higher drug levels). Mutant colonies, which were all smaller than those seen for strain 7785, were restreaked on plates containing the same level of PD 0305970. Four of the first-step mutants, 1QD1 to 1QD4, were analyzed further. Of these mutants, strain 1QD1 alone had an unstable resistance phe-

notype, and so mutant 1QD2 (MIC of 0.06 μ g/ml) was challenged with PD 0305970 at 0.125 μ g/ml, yielding second-step mutants 2QD1 to 2QD5. The third-step mutants 3QD1 to 3QD3, 3QD4 to 3QD7, and 3QD8 to 3QD11 were obtained by challenge of strain 2QD1 (MIC of 0.25 μ g/ml) with PD 0305970 at 1, 0.5, and 0.375 μ g/ml, respectively.

The mutant frequencies using 7785 and 1QD2 were 2×10^{-7} to 3×10^{-7} and 2×10^{-8} , respectively. Mutant frequencies using 2QD1 as parent and PD 0305970 at 1, 0.5, and 0.375 μ g/ml were 1×10^{-8} , 1×10^{-7} , and 1×10^{-7} , respectively.

PCR and DNA sequence analysis. PCR was employed to amplify DNA from the QRDRs of the gyrase and topo IV genes of PD0305970-selected *S. pneumoniae* mutants using the published PCR conditions and the following primers: H4025 and H4026 for *gyrB*, S6398 and S6399 for *parE*, VGA3 and VGA4 for *gyrA*, and M0363 and M4721 for *parC* (38). DNA was purified using Qiagen miniprep columns and sequenced directly by Lark Technologies using a series of nested primers. DNA sequences were compared to the published gyrase and topo IV gene sequences for parent strain 7785 (40, 43).

To exclude the possibility that other topoisomerase mutations could have contributed to quinazolinone resistance, we sequenced in full the *gyrA*, *gyrB*, *parC*, and *parE* genes of third-step mutant 3QD1 derived from the lineage 7785-1QD2-2QD1-3QD1. Full-length genes were amplified from chromosomal DNA using *Taq* polymerase and the following primer pairs: VGA35/VGA36 for *gyrA*, P7164/P7165 for *gyrB*, N6894/VPC2 for *parC*, and N7043/7044 for *parE* (43). PCR products were sequenced directly using a series of nested primers complementary to the templates. No mutations were detected with gyrase and topo IV genes, aside from the *gyrB* E474D and *parE* E475A changes (see Table 2).

Expression of GyrB E474D, GyrB E474A, and ParE E475A. Full-length mutant *gyrB* and *parE* genes were amplified using Vent DNA polymerase, template DNA from *S. pneumoniae* strains 3QD1 and 2QD2, and *gyrB* primers P7164/P7165 or *parE* primers N7043/7044 (43). The PCR products were processed, and the mutant *gyrB* and *parE* genes were cloned into pET19b vector using the same approach as that published for the wild-type (wt) genes (43). The topoisomerase genes in the resulting plasmids were sequenced to verify that the single desired mutation was present in the cloned gene. Overexpression plasmids were transformed into BL21(λ DE3) pLysS in preparation for protein expression. Culture induction and protein purification procedures using nickel-chelate chromatography were the same as those described previously for the wt GyrB and ParE proteins (43). The specific activities of GyrB, GyrB E474D, and GyrB E474A in DNA supercoiling were 5×10^4 , 4.4×10^4 , and 4.2×10^4 U/mg; the specific activity of ParE E475A in kDNA decatenation was 10^5 U/mg.

DNA supercoiling, decatenation, and cleavage assays. Methods and conditions for assaying DNA gyrase and topo IV catalytic activity, as well as DNA cleavage, have been described earlier (43). DNA reaction products were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and photographed and quantitated with an Alpha Innotech digital camera.

End-labeled DNA fragments and high-resolution mapping of gyrase cleavage sites. Oligonucleotide primers (30 pmol) were 5' end-labeled by mixing with 30 pmol of [γ - 32 P]ATP and T4 polynucleotide kinase. The reaction mixture was incubated at 37°C for 30 min and heat inactivated at 90°C for 3 min. The labeled primers were used to obtain PCR products 5' end-labeled on one or the other DNA strand and also to generate the fmol cycle sequencing DNA ladder.

A 290-bp S fragment from *S. pneumoniae* 7785 internal to the *parE* gene was used as a substrate for cleavage studies (27, 39). The S fragment was generated by PCR using forward primer S6398 and reverse primer S6399 (27). To obtain specifically labeled DNA fragments, PCR was set up with 10 pmol of [32 P]-labeled primer plus an equal amount of an unlabeled reverse primer.

Cleavage reactions were carried out in DNA supercoiling assay buffer (20 μ l) containing 300 fmol labeled DNA substrate, 2 μ g of GyrA and 4 μ g of GyrB, 1 mM ATP, 100 μ M gemifloxacin, or 50 μ M PD 0305970. Reaction mixtures were incubated for 1 h at 37°C, and then sodium dodecyl sulfate (SDS) and proteinase K were added to final concentrations of 1% and 100 μ g/ml, respectively. Incubation was continued for another hour at 42°C and was followed by ethanol precipitation. Samples were analyzed on a 6% sequencing gel.

Reversal of DNA cleavage. Supercoiled plasmid pBR322 (0.45 μ g) was incubated for 1 h at 37°C with *S. pneumoniae* GyrA (0.45 μ g) and GyrB (1.7 μ g) in the absence or presence of 2.5 μ M PD 0305970, 20 μ M gemifloxacin, or 80 μ M ciprofloxacin (total volume, 20 μ l). The reaction buffer contained 35 mM Tris-HCl (pH 7.5), 6 mM MgCl $_2$, 1.8 mM spermidine, 24 mM KCl, 5 mM dithiothreitol, 36 μ g/ml bovine serum albumin, 6.5% (wt/vol) glycerol. DNA cleavage was induced by addition of SDS to 1% and proteinase K treatment (100 μ g/ml) for 1 h at 42°C. Prior to cleavage induction, drug-containing reaction mixtures were incubated with 0.6 M NaCl for 15 min, with 35 mM EDTA for 15 min, or at 65°C for 15 min. DNA samples were analyzed by electrophoresis in a 1% agarose gel

TABLE 1. Quinolone-resistant *S. pneumoniae* mutants are susceptible to PD 0305790

Strain	Mutation in:		MIC ($\mu\text{g/ml}$) of ^a :	
	ParC	GyrA	PD 0305790	CIP
D5			0.03	1
7785			0.03	1–2
1C1 ^b			0.03	2–4
2C6	S79Y	None	0.06	16
2C7	S79F	None	0.06	16
1S1	None	S81F	0.03	1–2
1S4	None	S81Y	0.03	1–2
2S1	S79Y	S81F	0.06	64
3C4	S79Y	S81Y	0.06	128

^a CIP, ciprofloxacin; PD0305790, 3-amino-7-[(R)-3-[(S)-1-amino-ethyl]-pyrrolidin-1-yl]-1-cyclopropyl-6-fluoro-8-methyl-1H-quinazoline-2,4-dione.

^b Presumed efflux mutant, as CIP MIC was reduced in the presence of reserpine (21).

run in Tris-borate-EDTA buffer. The same conditions were used to study reversal in the presence of 1 mM ATP, except that the drug concentrations were reduced by two-thirds.

DNA unwinding. Relaxed pBR322 (0.8 μg) was mixed with topo I (calf thymus, 10 U) in 45 μl containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.5 mM dithiothreitol, 50 μM EDTA, 30 $\mu\text{g/ml}$ of bovine serum albumin, and MgCl_2 (50 μM to 24 mM). Samples were incubated at room temperature for 10 min, and 5 μl of 10-fold-concentrated ciprofloxacin or PD 0305790 was added. Solutions were then incubated at 37°C for 1 h. The reaction was stopped by addition of SDS (0.2% [wt/vol]), and samples were extracted with an equal volume of phenol and then with an equal volume of chloroform. DNA was precipitated with ethanol, dried, and redissolved in Tris-EDTA buffer (20 μl). DNA samples (10 μl) were analyzed by electrophoresis in 1% agarose gels run in Tris-phosphate-EDTA buffer in the absence or presence of chloroquine at 3 $\mu\text{g/ml}$. DNA was visualized by staining with ethidium bromide. In other unwinding experiments, the MgCl_2 concentration was fixed and a range of drug concentrations (10 to 320 μM) was tested.

RESULTS

PD 0305790 selects for novel *gyrB* and *parE* Toprim mutants of *S. pneumoniae*. Initial studies revealed that PD 0305790 was highly active against wt *S. pneumoniae* strains D5 and 7785 with MICs of 0.03 $\mu\text{g/ml}$ versus 1 to 2 $\mu\text{g/ml}$ for ciprofloxacin (Table 1). Significantly, the dione retained potency against isogenic 7785 mutants resistant to ciprofloxacin, including the *parC gyrA*

double mutants 2S1 and 3C4, whose ciprofloxacin MICs were 64 and 128 $\mu\text{g/ml}$, respectively (Table 1). As a first step in examining how PD 0305790 targets gyrase/topo IV, we isolated drug-resistant mutants of *S. pneumoniae* 7785. Consecutive drug challenges with PD 0305790 allowed recovery of first-step mutants, e.g., 1QD2 (bearing no topoisomerase changes); second-step *gyrB* (E474D, E474A, or R456H) mutants, e.g., 2QD1 to 4; and third-step *parE* (D435H or E475A) mutants, e.g., 3QD1 and 3QD4 (Table 2). First-, second-, and third-step mutants exhibited stepwise 2-fold, 4-fold, and 8- to 16-fold incremental changes in PD0305790 MICs, respectively (Table 2). Except for mutant 3QD4, which showed a two- to fourfold increase in the ciprofloxacin MIC, the second- and third-step mutants showed no increase in the MIC for ciprofloxacin and sparfloxacin. Indeed, most mutants displayed a two- to fourfold hypersusceptibility to quinolones, particularly sparfloxacin (Table 2). It appears that PD 0305790 selects for *gyrB* and then *parE* mutations that map in the Toprim domains.

Toprim mutations inhibit dione-stimulated DNA cleavage by gyrase. To gain functional information on PD 0305790 action, we expressed *S. pneumoniae* GyrB E474A, GyrB E474D, and ParE E475A subunits in *E. coli* and purified the proteins to >95% homogeneity by nickel-chelate chromatography (see Fig. S1 in the supplemental material). wt and mutant proteins were used to reconstitute gyrase (DNA supercoiling) and topo IV (DNA decatenation) activities, which were examined in enzyme inhibition and DNA cleavage assays. PD 0305790 was a highly potent inhibitor of wt *S. pneumoniae* gyrase and topo IV, inducing 50% enzyme inhibition (IC_{50}) at 2.5 μM and 1 μM (Fig. 2A and C), which can be compared with ciprofloxacin IC_{50} s of 80 and 10 μM , respectively (Table 3; gels not shown). GyrA S81F and ParC S79F mutations had little impact (less than twofold) on dione inhibition (Fig. 2B and D). In contrast, the GyrB E474D gyrase, GyrB E474A gyrase, and ParE E475A topo IV were resistant to dione inhibition (Fig. 2A and C), showing 16-fold, 8-fold, and 5-fold increases in the IC_{50} s, respectively, compared to those of the wt proteins (Table 3). These data provide direct evidence that these mutations affect dione action.

For DNA cleavage, the supercoiled pBR322 plasmid was

TABLE 2. *S. pneumoniae* mutants selected for resistance to PD 0305790 have changes in *gyrB* and/or *parE*

Strain	MIC ($\mu\text{g/ml}$) of ^a :			Mutation in the QRDR of the gene encoding ^b :			
	QD	CIP	SPX	GyrA	ParC	GyrB	ParE
7785	0.03	1–2	0.5				
1QD2 ^{c,d}	0.06	2	1	None	None	None	None
2QD1	0.25	2	0.25–0.5	None	None	E474D	None
2QD2 ^{c,e}	0.25	1	0.125–0.25	None	None	E474A	None
2QD4 ^f	0.25	0.25–0.5	0.125–0.25	None	None	R456H	None
3QD1 ^g	4	1	0.25	None	None	E474D	E475A
3QD4 ^h	2	4	0.5	None	None	E474D	D435H

^a QD, compound PD 0305790; CIP, ciprofloxacin; SPX, sparfloxacin.

^b The indicated GyrB mutations resulted from the following nucleotide changes: E474D, GAA→GTA; E474A, GAA→GCA; and R456H, CGC→CAC. The acquired ParE mutations resulted from the following nucleotide changes: E475A, GAG→GCG; and D435H, GAC→CAC.

^c Mutant strain used as the parent in the subsequent step of selection.

^d Drug susceptibility profile exhibited by the first-step mutants 1QD3 and 1QD4. No mutations were seen in the *gyrA*, *parC*, *gyrB*, or *parE* QRDRs of these strains.

^e Drug susceptibility profile shared by the second-step mutant 2QD3.

^f Drug susceptibility profile shared by second-step mutant 2QD5.

^g Drug susceptibility profile shared by third-step mutant 3QD2.

^h Drug susceptibility profile shared by third-step mutants 3QD5, 3QD10, and 3QD11.

FIG. 2. Inhibitory activity of PD 0305970 against wild-type and mutant *S. pneumoniae* type II topoisomerases. Inhibition of DNA supercoiling by DNA gyrase. Relaxed pBR322 plasmid DNA (0.4 μ g) was incubated with wild-type (wt) gyrase activity (1 U) or mutant enzyme (1 U) reconstituted with GyrB 474D or GyrB 474A (A) or GyrA S81F (B), in the presence of 1.4 mM ATP and the indicated concentrations (μ M) of PD 0305970. Reactions were stopped, and DNA species were separated by electrophoresis in 1% agarose. After staining with ethidium bromide, DNA was photographed under UV light. Lanes a and b are supercoiled and relaxed pBR322 DNA, respectively. Sc, R, and N denote supercoiled, relaxed, and nicked DNA, respectively. Inhibition of DNA decatenation by *S. pneumoniae* topo IV. Kinetoplast DNA (0.4 μ g) was incubated with wt topo IV (1U) or mutant enzyme (1 U) reconstituted with ParE 475A (C) or ParC 79F (D) and incubated with 1.4 mM ATP and PD 0305970 at the levels (μ M) indicated. DNA was separated and analyzed as described above. Lane a, kDNA. M indicates released minicircles.

TABLE 3. Potencies of PD 0305970 and ciprofloxacin against wt and mutant *S. pneumoniae* gyrase and topo IV^c

Enzyme	IC ₅₀ (μ M)		CC ₂₅ (μ M)	
	PD 0305970	CIP	PD 0305970	CIP
wt gyrase	2.5	80	0.5	40
GyrA + GyrB E474D	40	80	>40 ^a	40–80
GyrA + GyrB E474A	20	20	>40 ^b	40–80
GyrA S81F + GyrB	2.5–5	640	0.5	>640
wt topo IV	1	10	0.25	2.5–5
ParC + ParE E475A	5	2.5	10	2.5–5
ParC S79F + ParE	2.5	>160	0.5	>160

^a Only 5 to 10% of input pBR322 DNA was cleaved using PD 0305970 at 40 μ M.
^b Only 10% of DNA was cleaved at 40 μ M.
^c CIP, ciprofloxacin.

incubated with DNA gyrase or topo IV complexes in the absence or presence of increasing concentrations of PD 0305970 or ciprofloxacin. DNA breakage was induced by addition of SDS and proteinase K, and the DNA was examined by agarose gel electrophoresis. Figure 3 shows data obtained with PD 0305970 in the absence of ATP. The dione promoted efficient gyrase- and topo IV-mediated single- and double-stranded breakage of pBR322 with drug concentrations that convert 25% of the input DNA to the linear form (CC₂₅s) of 0.5 and 0.25 μ M, respectively (Fig. 3A and C; Table 3). These values are 80- and 10- to 20-fold lower than the ciprofloxacin CC₂₅s, respectively (Table 3). Though the GyrA S81F and ParC S79F mutations were highly effective in blocking cleavage complex formation with ciprofloxacin (CC₂₅s of >640 and >160 μ M) (45, 57; data not shown), they had little (less than twofold) effect on the dione CC₂₅s (Fig. 3B and D; Table 3). In contrast, mutant

FIG. 3. Capture of gyrase and topo IV cleavage complexes by PD 0305970. GyrB 474D and 474A (A) and ParE 475A (C) changes (but not GyrA 81F [B] and ParC 79F [D] mutations) inhibit DNA cleavage by *S. pneumoniae* gyrase and topo IV. (A and B) Supercoiled plasmid pBR322 DNA (0.4 μ g) was incubated with either wt or mutant *S. pneumoniae* gyrase reconstituted from appropriate GyrB (1.7 μ g) and GyrA subunits (0.45 μ g) in the presence of PD 0305970 at the concentrations (μ M) indicated. Cleavage was induced by addition of SDS and after proteinase K treatment; DNA was examined by electrophoresis in 1% agarose. Lanes a and b, supercoiled pBR322 and EcoRI-linearized pBR322. N, L, and Sc denote nicked, linear, and supercoiled DNA, respectively. (C and D) Cleavage by *S. pneumoniae* topo IV was conducted as for gyrase (A), but by using the respective ParE (1.7 μ g) and ParC (0.45 μ g) proteins. Samples were analyzed as described for DNA gyrase.

enzymes bearing GyrB E474D, GyrB E474A, and ParE E475A mutations exhibited dione CC_{25} values of >40 , >40 , and 10 μ M, respectively, i.e., >80 -fold, >80 -fold, and 40-fold higher, respectively, than those of the wt enzymes (Table 3). It can be concluded that the GyrB and ParE changes identified in dione-resistant *S. pneumoniae* are highly effective in blocking DNA cleavage promoted by the dione, whereas quinolone action was largely unaffected.

It is known that ATP can stimulate quinolone-promoted DNA cleavage. To test the effects of ATP on PD 0305970-promoted DNA cleavage by wt and mutant gyrase and topo IV, we also carried out the same experiments shown in Fig. 3, but in the presence of 1 mM ATP (see Fig. S2 in the supplemental material). As expected, in the absence of drug, ATP inhibited gyrase relaxation of the supercoiled pBR322 substrate (seen weakly in Fig. 3A and B; see also Fig. S2A and B in the supplemental material) and conversely promoted complete plasmid relaxation by topo IV (see Fig. S2C and D in the supplemental material). Inclusion of ATP did not markedly affect the efficiency of DNA cleavage for either enzyme and its mutants (twofold reduction in CC_{25} for wt gyrase), with the exception of the GyrB474A mutant

gyrase whose CC_{25} was reduced from >40 to 10 μ M (see Fig. S2A in the supplemental material).

We note that E474 and E475 are acidic residues that might conceivably bind Mg^{2+} . However, the wt, GyrB 474A, and ParE 475A enzymes exhibited similar specific activities, Mg^{2+} dependence and Ca^{2+} requirement in Ca^{2+} -dependent DNA cleavage (K. Gould and L. M. Fisher, data not shown). Thus, it seems unlikely that these residues are directly involved in metal binding.

Repertoire of dione-induced DNA breakage sites shares partial overlap with quinolones. Previous studies have shown that quinolones trap topoisomerase cleavage complexes at specific sites on DNA in a reaction requiring Mg^{2+} ions. Although the disposition of Mg^{2+} ions in the cleavage complex is incompletely understood, it is known that quinolones chelate Mg^{2+} via their 3-carboxy-4-keto moiety and bind DNA in a Mg^{2+} -dependent fashion (26, 37, 48). PD 0305970 differs from quinolones in carrying a 3-amino 2,4-dione system that might affect ion chelation and DNA cleavage specificity. To examine this issue, we carried out DNA cleavage with gyrase using linear plasmid pBR322 as a substrate in the presence of PD 0305970

FIG. 4. PD 0305970 induces site-specific DNA cleavage by gyrase. (A) PD 0305970 promotes gyrase-mediated double-stranded DNA cleavage. pBR322 DNA linearized with EcoRI was employed as a substrate in a cleavage assay with wild-type *S. pneumoniae* gyrase (as described in the legend for Fig. 3) by using PD 0305970 (QD) or ciprofloxacin (CIP) at the concentrations indicated. Lane M, DNA markers; molecular sizes in kilobase pairs (Kb) are indicated on the left. (B) Dione-induced DNA cleavage by gyrase analyzed at the sequence level. The 290-bp S fragment from the *S. pneumoniae* *parE* gene (27) was labeled with 33 P at the 5' end of the top strand and incubated with *S. pneumoniae* gyrase and 1 mM ATP in the absence or presence of 100 μ M gemifloxacin (Gemi) or 50 μ M PD 0305970 (QD). After cleavage induction with SDS and proteinase K, DNA was precipitated with ethanol and run on a 6% denaturing polyacrylamide gel alongside ACGT chain termination sequencing products. Filled and open arrows indicate DNA fragments induced specifically by QD and gemifloxacin, respectively.

or ciprofloxacin (Fig. 4A). Cleavage at any particular site on the DNA is expected to produce two discrete fragments. The dione promoted dose-dependent DNA cleavage at specific sites to yield a pattern of cleavage fragments that was similar, but not identical, to that observed for ciprofloxacin. The two agents also generated similar spectra of DNA cleavage fragments with *S. pneumoniae* topo IV, and like gyrase, the reaction with topo IV required the presence of Mg^{2+} ions (X.-S. Pan and L. M. Fisher, data not shown).

To examine cleavage specificity at the nucleotide level, gyrase-mediated dione- and quinolone-induced cleavage was carried out in the presence of 1 mM ATP using the *S. pneumoniae* *parE* S fragment specifically 33 P-labeled at one 5' end. This DNA substrate has been used previously to analyze site-specific DNA cleavage by gyrase induced by gemifloxacin, an antipneumococcal quinolone (27) and generated the expected pattern of DNA fragments (Fig. 4B). PD 0305970 also induced site-specific DNA cleavage to produce fragments that comigrated with chain termination DNA sequencing products (and some quinolone-induced fragments) (Fig. 4B), consistent with the presence of 3' OH ends. Further analysis revealed that the dione promoted DNA cuts on complementary DNA strands that were separated by a 4-bp stagger, another characteristic feature of gyrase (X.-S. Pan and L. M. Fisher, data not shown). Interestingly, although some dione-stabilized cleavage products were also induced by gemifloxacin (Fig. 4B), many fragments were specific to the dione or to gemifloxacin. Thus, PD 0305970-induced DNA cleavage by gyrase has a distinct sequence specificity, producing a repertoire of sites that only partially overlaps that observed with gemifloxacin.

Dione-stabilized cleavage complexes are not fully reversed by EDTA. A characteristic feature of cleavage complexes stabilized by most quinolones is their full reversal by high salt, EDTA, or heat (39). To examine the reversibility of dione-induced cleavage, we incubated gyrase with supercoiled pBR322 in a cleavage assay in the absence of ATP and the presence of 2.5 μ M PD 0305970, 20 μ M gemifloxacin, or 80 μ M ciprofloxacin, i.e., concentrations that promote similar levels of DNA cleavage (Fig. 5, top). When drug was omitted, there was no DNA cleavage, just the well-documented ATP-independent relaxation by gyrase of supercoiled DNA (Fig. 5, cf. lanes a and 0). Inclusion of drug generated nicked DNA, full-length linear DNA, and a smear of smaller linear fragments arising from two or more breaks per plasmid (Fig. 5, lane C). For ciprofloxacin, prior incubation for 15 min with 0.6 M NaCl, 35 mM EDTA, or heating at 65°C reversed DNA scission, allowing recovery of supercoiled DNA substrate. Though full EDTA and heat reversal was observed for gemifloxacin, it was found (unusually for quinolones) that salt reversal was inefficient judged by the continued recovery of linear DNA (Fig. 5), probably reflecting the tight binding of this high-affinity quinolone (56). Interestingly, for PD 0305970-stabilized complexes, only heat caused full reversal of DNA breakage; neither salt nor EDTA was effective. The differential responses to EDTA, a Mg^{2+} chelator, could suggest that the drugs interact differently with the cleavage complex.

To investigate the effects of ATP on cleavage reversibility, the experiment shown in Fig. 5 (top) was carried out in the presence of 1 mM ATP and using one-third the concentration of each drug to ensure comparable cleavage levels between the

FIG. 5. Reversal of drug-stabilized DNA cleavage in the absence (top) or presence (bottom) of ATP. (Top) Supercoiled plasmid pBR322 (0.45 μ g) was incubated with *S. pneumoniae* GyrA (0.45 μ g) and GyrB (1.7 μ g) in a DNA cleavage assay in the presence of 2.5 μ M PD 0305970 (QD), 20 μ M gemifloxacin (Gemi), or 80 μ M ciprofloxacin (CIP). After addition of SDS and proteinase K, DNA samples were purified and analyzed by electrophoresis in 1% agarose. Prior to cleavage induction, samples were incubated with 0.6 M NaCl for 15 min (lanes S), with 35 mM EDTA for 15 min (lanes E), or at 65°C for 15 min (lanes H). Lanes C, no pretreatment prior to cleavage; lane 0, cleavage with gyrase in the absence of drug; lanes a and b, supercoiled and linear pBR322, respectively. N, L, and Sc denote nicked, linear, and supercoiled DNA, respectively. (Bottom) Experiments were performed as described in the legend for the top panel, except 1 mM ATP was included and the drug concentrations were reduced to 0.83 μ M PD 0305970, 6.7 μ M gemifloxacin, and 26.7 μ M ciprofloxacin.

experiments (Fig. 5, bottom). Inclusion of ATP had no obvious effects on cleavage reversibility.

PD 0305970 does not promote Mg^{2+} -dependent DNA unwinding, in contrast to quinolones. The outcome of reversal experiments (Fig. 5) led us to examine whether PD 0305970 is able to bind and unwind double-stranded DNA in a Mg^{2+} -dependent fashion, a known feature of quinolones (37). Relaxed pBR322 plasmid DNA was incubated with increasing levels of $MgCl_2$ (50 μ M to 8 mM) and calf thymus topo I (to remove positive supercoils generated by unwinding) in the absence or presence of PD 0305970 or ciprofloxacin. Reactions were stopped by addition of SDS, the drugs were removed by extraction with phenol, and the DNA products were displayed by agarose gel electrophoresis carried out in the absence (CQ $^-$) or presence (CQ $^+$) of chloroquine, a DNA intercalator that induces positive supercoiling of DNA (Fig. 6). Relaxation of DNA in the presence of increasing Mg^{2+} levels led to a slight increase in positive supercoiling of the DNA compared to that with the no- Mg^{2+} control (Fig. 6A, CQ $^+$ and $-QD$ lanes), consistent with the known effects of Mg^{2+} on DNA helix pitch. Inclusion of 160 μ M PD 0305970 had little effect on the topoisomer distribution at any metal ion concentration (Fig. 6A, $+QD$ lanes). In contrast, inclusion of 160 μ M ciprofloxacin (Fig. 6B, CQ $^-$) caused DNA unwinding (reduced mobility in the CQ $^+$ gel) that was maximal in the presence of 0.5 mM Mg^{2+} and disappeared at higher Mg^{2+} concentrations. A similar "spike" in DNA unwinding has been reported for norfloxacin (37) and has been attributed to DNA unwinding through the formation and binding of a quinolone- Mg^{2+} complex and competitive inhibition of its DNA binding by high Mg^{2+} levels. Our results indicate that unlike quinolones, PD 0305970 does not interact with Mg^{2+} to induce helix unwinding, a potentially important difference for cleavage complex formation with topoisomerases.

DISCUSSION

We have shown that PD 0305970, a novel quinazolinone, targets gyrase in *S. pneumoniae* and promotes double-stranded DNA breakage by gyrase and topo IV in vitro. By genetic and biochemical approaches, we demonstrate that dione-stabilized DNA breakage has unique features, including strong involvement of the GyrB (ParE) Toprim domain, tolerance of quinolone-resistance GyrA (ParC) changes, and differences in DNA sequence specificity, reversibility and metal ion interactions that discriminate it from enzyme poisoning by quinolones. Thus, although diones and quinolones bear structural similarity, there are clear differences in target enzyme responses that have implications for the mechanism and the circumvention of quinolone resistance.

Preliminary studies suggested that PD 0305970 targets both gyrase GyrB and topo IV ParE in *S. pneumoniae* (23). This conclusion was based on analysis of just two mutants selected for with PD 0305970: one bearing an alteration in *gyrB* (D435E) and a second mutant with multiple changes, i.e., *gyrB* (D435E and E475A) *parE* (P454S). By a more systematic analysis, we recovered a number of stepwise mutants in which a single mutation in *gyrB* (E474D, E474A, or R456H) was followed by a single mutation in *parE* (E475A or D435H) associated with stepwise 4-fold and 8- to 16-fold increases in the dione MIC (Table 2). Moreover, we could show that gyrase and topo IV bearing the respective GyrB E474A/D and ParE E475A subunits were resistant to dione inhibition and dione-induced DNA cleavage (Fig. 2A and C and 3A and C). These results provide the first functional evidence linking mutations to dione resistance (Table 3). Our data showing that PD 0305970 selects for the *gyrB* mutant and then the *parE* mutant suggest that gyrase is the primary topoisomerase target and that topo IV is a secondary target of the dione in *S. pneumoniae*, as observed

FIG. 6. Assay for DNA unwinding of closed circular DNA by PD 0305970 and ciprofloxacin. Relaxed pBR322 DNA (0.8 μ g) was preincubated with calf thymus DNA topo I (10 U) at room temperature for 10 min in the presence of $MgCl_2$ at the concentrations (mM) indicated. After the addition or omission of PD 0305970 (QD) (A) or ciprofloxacin (CIP) (B), each at 160 μ M, reaction mixtures were further incubated at 37°C for 1 h, and then the topo I was inactivated. Samples were extracted with phenol to remove bound ligand, and DNA products were separated and analyzed by electrophoresis in Tris-phosphate-EDTA buffer. Half of each DNA sample was analyzed in the absence of chloroquine (CQ-); the other half was subjected to electrophoresis in the presence of chloroquine (CQ+). In the absence of drugs, DNA samples are slightly positively supercoiled and are made more positively coiled by inclusion of chloroquine in the gel. Lanes a, untreated relaxed pBR322 DNA employed as substrate. QD and CIP were added from stock solutions in 0.1 M NaOH and in DMSO, and their concentrations were adjusted in each reaction to 10 mM and 2.5% (vol/vol), respectively.

for several quinolones (1, 17, 41). This genetic assignment of gyrase as the primary dione target contrasts with the slightly greater dione sensitivity of topo IV over gyrase seen in enzyme assays (Table 3). Possibly differences in the intracellular conditions coupled with the threefold greater cellular levels of gyrase over those of topo IV (44) could explain the primacy of gyrase as the dione target.

All five *gyrB* and *parE* mutations that we identified by mutant selection are novel, and (along with earlier mutations [23]) all map to a short region of the Toprim domain which we denote the “dione resistance-determining region” (DRDR) and which overlaps the QRDR (Fig. 7). Indeed, the *ParE* D435H and *GyrB* R456H changes lie directly within the highly conserved EGDSA and P(L/I)RGK motifs (residues shown in bold), whose alteration (e.g., D to N and K to E) in *E. coli* was originally linked to quinolone resistance, i.e., 4- to 16-fold increases in the MIC (58). For *S. pneumoniae*, specific mutations in the EGDSA motif (D to E or V in *GyrB*, D to N in *GyrB* and *ParE*) and E474K (in *GyrB* and *ParE*) have been reported in quinolone-resistant strains (21, 42, 45, 53). Genetic studies reveal that several of these changes confer at most approximately twofold increases in resistance to some quinolones (53) and, we note, are largely different from those selected for with PD 0305970 (Fig. 7). One interesting aspect to

emerge from our mutant selection is that many DRDR mutations and especially PLRGK (residue shown in bold) (R to H) actually conferred hypersusceptibility to quinolones (Table 2), a phenotype reported previously for nalidixic acid-selected PL-

FIG. 7. Resistance to PD 0305970 maps to residues in the Toprim domain of the *S. pneumoniae* *GyrB* and *ParE* proteins. Filled arrows denote quinazolinodione resistance mutations described here; unfilled arrows denote those described by Huband et al. (23). Boldface type highlights conserved EGDSA, P(I/L)RGK, and (I/L)MTDXDXD motifs containing residues (underlined) implicated in Mg^{2+} ion binding. Residues 431 to 510 of the *GyrB*/*ParE* protein (40) are shown.

RGK (K to E) mutants of *E. coli* (58). Hypersusceptibility to some quinolones, but resistance to others, was attributed to altered interactions of the mutant GyrB, with the different drug substituents at C-7 causing stabilization or destabilization of the drug complex (58). According to this view, very strong affinity between DRDR residues and the C-7 moiety of the drug could explain the enhanced potency of PD 0305970 vis-à-vis quinolones (Tables 1 and 3), selection for DRDR mutants, and overlap with the QRDR (Fig. 7).

An interesting feature of the GyrB DRDR/QRDR is that residues (underlined) in the EGDSA motif together with the downstream (L/I)MTDADXD sequence are also implicated in binding Mg^{2+} , an essential cofactor for DNA scission (Fig. 7) (34, 54). In fact, the yeast topo II-DNA structure reveals a Mg^{2+} ion coordinated by these conserved glutamate and aspartate residues lying close to the catalytic tyrosine provided by the breakage-reunion domain and positioned with one for each DNA strand (12). Overlap of the GyrB DRDR and QRDR with the Mg^{2+} binding region of the Toprim domain and the presence of the GyrA QRDR in the breakage-reunion domain (24, 31) could suggest that diones and quinolones might interact with Mg^{2+} .

Several lines of evidence suggest that gyrase makes different contacts with the diones and quinolones. First, exclusive selection for Toprim mutants by PD 0305970 rather than GyrA/ParC mutants plus the absence of cross-resistance with quinolones (Table 1 and 2) indicate differences in side-chain involvement. The beneficial upshot is that *gyrA/parC* quinolone resistance mutations do not impact dione-trapping of the cleavage complex (Fig. 2B and D and 3B and D; Table 3), explaining dione activity against quinolone-resistant mutants (Table 1) (23). On the other hand, PD 0305970 may be less active against established *gyrB* and *parE* quinolone resistance mutants, though these are encountered less frequently as clinical isolates. Second, it is known that quinolones bind DNA in a Mg^{2+} -dependent manner, with a preference for single-stranded DNA, especially polyguanine (27, 37, 48). Differences in DNA cleavage specificity (Fig. 4) indicate that the structural features of PD 0305970 are decoded differently at the gyrase-DNA gate compared to those of quinolones. Third, the lack of EDTA reversal of PD 0305970-induced gyrase cleavage is in marked contrast to cleavage reversal for gemifloxacin and ciprofloxacin, a hallmark of quinolone drugs (Fig. 5).

One or all of these effects likely involves the 3-amino-2,4-dione moiety of PD 0305970 that replaces the Mg^{2+} -coordinating 3-carboxy-4-keto grouping of the quinolone (Fig. 1). The bidentate dione could form novel hydrogen bonding interactions with the enzyme or DNA, accounting for different domain preferences in mutant recovery and DNA cleavage. Alternatively, unlike quinolones, which form a 1:1 complex with Mg^{2+} (26), conceivably the dione could complex two Mg^{2+} ions, forming a very tight complex and thereby blocking cleavage reversal by EDTA (Fig. 5). However, we note that Mg^{2+} did not mediate DNA unwinding by PD 0305970, in contrast to the significant unwinding observed for ciprofloxacin (Fig. 6). It is believed that Mg^{2+} forms a coordination complex with quinolone and DNA that stabilizes drug-nucleic acid interactions, allowing detection in the unwinding assay (37). Lack of DNA unwinding and EDTA reversal could suggest altered (or absent) metal ion coordination by the dione. Interestingly, its des-3-amino analogue PD 0326448 is almost as

active an antibacterial agent as PD 0305970 (23), demonstrating that the 3-amino group is functionally dispensable, although this derivative does retain a bidentate urea structure. Direct biophysical measurements are warranted to determine if diones do bind metal ions and DNA and to establish the role of such binding (if any) in cleavage complex stabilization.

Currently, there is no X-ray crystal structure of a dione-DNA-gyrase complex that would provide a framework with which to interpret our biochemical data. However, the crystal structure of the moxifloxacin-DNA cleavage complex of topo IV gives useful insight (25). In this structure, two quinolones are intercalated between the +1 and -1 nucleotides at the ends of the 4-bp staggered break formed by cleavage on each strand and stacked against the +1 purine bases. The N-1 cyclopropane ring of the drug lies close to the ParC helix α 4 helix residues S79 and D83, whose mutation causes resistance to quinolones. The bulky C-7 group is stacked on the DNA base at the +4 position of the complementary strand projecting out into solvent-accessible space in close proximity to ParE R456 of the PLRGK motif with D435 of the EGDSA farther away, as are ParE residues E474 and E475. Thus, Toprim residues conserved between topo IV and gyrase (Fig. 7) and whose mutation confers dione resistance (Table 2) lie within the vicinity of the quinolone C-7 substituent. Conceivably, an altered interaction of the dione vis-à-vis quinolones with the ParC α 4 helix region might orient the drug away from ParC residues that afford quinolone resistance and toward the Toprim domain. Further studies of the structural side will be required to understand and optimize the antimicrobial properties of these novel dione agents.

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