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Target-Based Resistance in *Pseudomonas aeruginosa* and *Escherichia coli* to NBTI 5463, a Novel Bacterial Type II Topoisomerase Inhibitor

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In a previous report (T. J. Dougherty, A. Nayar, J. V. Newman, S. Hopkins, G. G. Stone, M. Johnstone, A. B. Shapiro, M. Cronin, F. Reck, and D. E. Ehmann, Antimicrob Agents Chemother 58:2657–2664, 2014), a novel bacterial type II topoisomerase inhibitor, NBTI 5463, with activity against Gram-negative pathogens was described. First-step resistance mutations in *Pseudomonas aeruginosa* arose exclusively in the *nfxB* gene, a regulator of the MexCD-OprJ efflux pump system. The present report describes further resistance studies with NBTI 5463 in both *Pseudomonas aeruginosa* and *Escherichia coli*. Second-step mutations in *P. aeruginosa* arose at aspartate 82 of the gyrase A subunit and led to 4- to 8-fold increases in the MIC over those seen in the parental strain with a first-step *nfxB* efflux mutation. A third-step mutant showed additional GyrA changes, with no changes in topoisomerase IV. Despite repeated efforts, resistance mutations could not be selected in *E. coli*. Genetic introduction of the Asp82 mutations observed in *P. aeruginosa* did not significantly increase the NBTI MIC in *E. coli*. However, with the aspartate 82 mutation present, it was possible to select second-step mutations in topoisomerase IV that did lead to MIC increases of 16- and 128-fold. As with the gyrase aspartate 82 mutation, the mutations in topoisomerase IV did not by themselves raise the NBTI MIC in *E. coli*. Only the presence of mutations in both targets of *E. coli* led to an increase in NBTI MIC values. This represents a demonstration of the value of balanced dual-target activity in mitigating resistance development.

Gram-negative infections are currently a focus of increased concern in the medical community. The options for treatment of these pathogens have narrowed as a myriad of resistance mechanisms have emerged to challenge virtually every class of existing antibiotic (1–4). At the same time, attempts to identify fundamentally new targets and effective novel compounds to control multidrug-resistant (MDR) Gram-negative infections have largely fallen short of their goals. Although many novel compounds have been found in high-throughput screening (HTS) campaigns, it has proven extremely difficult to advance these to leads that can progress to the clinical testing phase. While some promising compounds, such as ceftazidime-avibactam, ceftolozane-tazobactam, plazomicin, and eravacycline, are in the late stages of clinical development (5–7), ongoing effective control of infections caused by MDR Gram-negative pathogens will demand additional new therapeutic compounds.

Given the failure of several novel target efforts to develop compounds that are effective in patients (8, 9), one strategy that has emerged is to identify new compounds that engage existing validated antimicrobial targets. There have been several reports of novel, nonquinolone compounds that interact with bacterial type II topoisomerases gyrase and topoisomerase IV (TopoIV); however, these are almost exclusively active primarily against Gram-positive pathogens, although one class of tricyclic compounds has broader activity (10–17). We have recently reported on a chemical class of topoisomerase inhibitors with activity against several Gram-negative multidrug resistant pathogens; these compounds have been termed novel bacterial type II topoisomerase inhibitors (NBTI) (11, 18). As previously reported, these compounds inhibit the two topoisomerase targets in the bacterial cell, gyrase and topoisomerase IV, in a manner distinct from that of the fluoroquinolone class, with inhibition not resulting in a cleavage complex (19, 20). The NBTI compounds were also found to maintain MIC activity against strains with topoisomerase mutations that impair fluoroquinolone target inhibition (18). In the initial report, we identified first-step resistance mutations in *Pseudomonas aeruginosa* exclusively in the *nfxB* regulator gene controlling the expression of the MexCD, OprJ efflux pump system (21). Subsequently, additional resistance development studies were performed to obtain a more detailed understanding of the NBTI compound resistance mechanisms.

In the present report, we describe target-based resistance mutations in the type II topoisomerases of both *P. aeruginosa* and *Escherichia coli* that affect susceptibility to NBTI 5463. These mutations were mapped in the target genes and found to be located on sites in the target enzymes that are distinct from the amino acid changes observed in fluoroquinolone resistance mutations. An interesting finding reported herein is that in the case of *E. coli*, single-target mutations in either gyrase or topoisomerase IV did not result in a significant increase in the MIC to the NBTI compound. Only after the introduction of mutations in both target enzymes was an increase in the MIC observed.

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TABLE 1 Strain details

Strain	Genotype
P. aeruginosa PA01	
AZ301	4-bp deletion from bp 490 to 493 in *nfxB*
AZ391	D82E in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ392	D82E in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ393	D82E in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ394	D82N in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ395	D82N in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ396	D82G in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ397	D82E in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ398	D82N in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ789	D82E and D87Y in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ790	D82E and D87Y in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ791	D82E and D87Y in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ792	D82E and D87Y in GyrA, 4-bp deletion from bp 490 to 493 in *nfxB*
AZ799	AZ392 with pMMB67
AZ800	AZ392 with pMMB67::*nfxB*
AZ801	AZ394 with pMMB67
AZ802	AZ394 with pMMB67::*nfxB*
AZ803	AZ396 with pMMB67
AZ804	AZ396 with pMMB67::*nfxB*
E. coli W3110	
AZ480	D82E in GyrA, *yfaL* is replaced by *kan*
AZ481	D82G in GyrA, *yfaL* is replaced by *kan*
AZ484	D82E in GyrA, K277N and K278L in ParC, *yfaL* is replaced by *kan*
AZ486	D82E in GyrA, P439Q in ParE, *yfaL* is replaced by *kan*
AZ489	D82G in GyrA, D79G in ParC, *yfaL* is replaced by *kan*
AZ530	D82E in GyrA, K277N and K278L in ParC, markerless deletion of *yfaL*
AZ674	D82E in GyrA, K277N and K278L in ParC, markerless deletion of *yfaL*, pKD46 (23) is present
AZ687	D82E in GyrA, K277N and K278L in ParC, markerless deletion of *yfaL*, *ygiU* is replaced by *kan*
AZ699	K277N and K278L in ParC, markerless deletion of *yfaL*, *ygiU* is replaced by *kan*
AZ704	D82E in GyrA, D79G in ParC, markerless deletion of *yfaL*
AZ711	D82E in GyrA, D79G in ParC, markerless deletion of *yfaL*, pKD46 is present
AZ717	D82E in GyrA, D79G in ParC, markerless deletion of *yfaL*, *ygiU* is replaced by *kan*
AZ727	D79G in ParC, markerless deletion of *yfaL*, *ygiU* is replaced by *kan*

MATERIALS AND METHODS

Strains and media. Experiments to generate resistant strains for characterization were performed with *P. aeruginosa* PAO1 and *E. coli* W3110. Experiments for resistant mutant selection and characterization were performed in LB broth and on LB agar. Susceptibility testing was performed in cation-adjusted Mueller-Hinton broth according to Clinical and Laboratory Standards Institute (CLSI) guidelines (22). A complete list of the strains employed in this study is presented in Table 1.

Resistant mutant selection. *P. aeruginosa* PAO1 (AZ301) and *E. coli* W3110 were both grown overnight in LB broth with shaking at 37°C. The *P. aeruginosa* PAO1 strain designated AZ301 had a 4-bp deletion in the *nfxB* coding region (18), leading to an MIC increase against the NBTI compound over that of the baseline PAO1 parent strain. For selection, 2 ml of cells from an overnight culture were centrifuged, resuspended in 0.5 ml of LB, and plated on 1×, 2×, 4×, and 8× MIC of NBTI 5463 on large-diameter (150-mm) plates. In addition, 10-fold serial dilutions of the liquid cultures were plated on antibiotic-free LB agar to determine the total number of bacteria. Plates were examined for potentially resistant colonies after 24 and 48 h of incubation at 37°C.

Gene amplification and sequencing. The genes for the gyrase and TopoIV subunits GyrA, GyrB, ParC, and ParE were amplified from *P. aeruginosa* and *E. coli* genomic DNA using previously described methods (18). DNA primers employed are listed in Table S1 in the supplemental material. Dideoxy DNA sequencing was performed using an Applied Biosystems 3100 series genetic analyzer.

Introduction of Asp82Glu and Asp82Gly in GyrA of *E. coli*. Wild-type *gyrA* was cloned into pET-46 EK/LIC (EMD Millipore, Billerica, MA) to obtain pJT596. pAN125 and pAN126 were obtained by introducing the missense mutations Asp82Glu and Asp82Gly into pJT596 by site-directed mutagenesis using the QuikChange kit from Agilent Technologies (Santa Clara, CA). The first 500 bp of the *gyrA* gene were amplified from pAN125 and pAN126. The PCR products were transformed into *E. coli* BW25113 (23, 24). Two different isolates with the Asp82Glu or Asp82Gly mutation encoded in *gyrA* were selected at a concentration of 1× MIC of NBTI5463 in the BW25113 background and sequenced to confirm the presence of the mutations. Since the change in MIC of these mutants to NBTI5463 was found to be minimal, to readily move these *gyrA* mutations to a clean genetic background in W3110 via P1 phage transduction, strains with a kanamycin resistance gene in adjacent nonessential genes were constructed.

Construction of the kanamycin-resistant strains for P1 phage cotransduction. P1 cotransduction relies on proximity of the genes. Hence, the *yfaL* gene, located adjacent to the *gyrA* gene (Fig. 1), was replaced by a kanamycin-resistant (Kan^r) gene (23) in strains containing the Asp82Glu and Asp82Gly missense mutations encoded in *gyrA*. P1 cotransduction was performed to move the tightly linked Kan^r gene and Asp82 mutations in *gyrA* into W3110 to obtain AZ480 and AZ481 (25, 26) by selecting for kanamycin resistance. The proximity of the Kan^r marker to the *gyrA* gene yielded greater than 95% cotransduction of the two genes with the P1 transducing phage. The cotransduction of the *gyrA* mutation with kanamycin resistance was confirmed by sequencing.

Mobilizing *parC* mutations in W3110. Strains AZ484 and AZ489 were used to mobilize *parC* mutations in *E. coli* W3110. The first step was to delete the Kan^r gene from *yfaL* using PCP20 (23) for flippase-mediated excision from AZ484 and AZ489. Subsequently, the open reading frame (ORF) of *ygiU* (selected due to its proximity to the *parC* gene) (Fig. 1) was replaced by the Kan^r gene for cotransduction of *parC* mutations along with kanamycin resistance into the W3110 background. Thus, strains AZ687 and AZ717 were created, containing the Kan^r gene in *ygiU* in addition to the *gyrA* and *parC* mutations. Two separate P1 lysates were prepared from AZ687 and AZ717 to transduce W3110 (24). Thus, strains with only ParC mutations were obtained: AZ699 (Lys277Asn and Lys278Leu encoded in *parC*) and AZ727 (Asp79Gly encoded in *parC*). Strain genotypes are listed in Table 1.

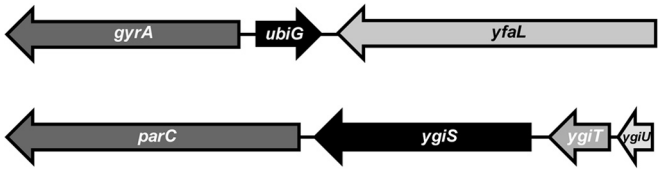

FIG 1 Closely linked genes are highly cotransduced by P1 phage generalized transduction. The kanamycin resistance genes were placed in *yfaL* and *ygiU*. (Top) Gene context for *E. coli gyrA* and *yfaL*, spanning approximately 6 kb. (Bottom) Gene context for *E. coli parC* and *ygiU*, spanning roughly 4 kb. P1 cotransduction of these closely linked genes exceeds 95%.

TABLE 2 Resistance rates of second-step *P. aeruginosa* mutants derived from first-step *nfxB* mutant

Concn, × MIC (8 μg/ml)	Mutation rate
1×	$>5.4 \times 10^{-9}$
2×	1.1×10^{-9}
4×	5.0×10^{-10}
8×	$<5.4 \times 10^{-10}$

TABLE 3 MICs and sequence data for gyrase A second-step *P. aeruginosa* mutants

Strain[a]	GyrA mutation	MIC (μg/ml)		
		NBTI 5463	Ciprofloxacin	Levofloxacin
PAO1		0.5	0.13	0.5
AZ301 (*nfxB* Δ490–493)		16	1	2
AZ391	D82E	256	1	2
AZ392	D82E	256	1	2
AZ393	D82E	256	1	2
AZ394	D82N	256	8	16
AZ395	D82N	256	8	16
AZ396	D82G	128	2	4
AZ397	D82E	128	1	2
AZ398	D82N	256	8	16

[a] AZ391, -392, -393, and -394 were isolated at 2× MIC (32 μg/ml). AZ395, -396, -397, and -398 were isolated at 4× MIC (64 μg/ml).

Mutant topoisomerase production. The gene sequences encoding *E. coli* ParC (1 to 752) Asp79Gly and *E. coli* GyrA (1 to 875) Asp82Gly were synthesized with an N-terminal 6×His tag and a tobacco etch virus (TEV) protease cleavage recognition sequence (GenScript, Piscataway, NJ). The genes were cloned into a modified version of the pET-28b expression plasmid. The plasmids were designated pJT984 and pJT985, respectively. Purification of mutant proteins followed methods similar to those used for the wild-type proteins (13, 27), employing a column chromatography sequence of immobilized metal affinity chromatography, followed by size exclusion chromatography. The His_6 and TEV purification tags were not removed, and protein purities were verified by SDS-PAGE and liquid chromatograph-mass spectrometry (LC-MS).

Topoisomerase activity assays. Measurement of catalytic activity and compound inhibition of the *E. coli* wild-type and mutant gyrase and TopoIV enzymes was performed using assays that detect the formation of phosphate from DNA-dependent ATP hydrolysis (13, 28).

RESULTS

Resistance in *P. aeruginosa*. We previously reported that the first-step resistance mutations identified in *P. aeruginosa* with the NBTI compounds were all found to be located in the *nfxB* regulator for the MexCD-OprJ efflux system (18). Using one of these strains, which contained a 4-bp deletion (490 to 493) in the 564-bp *nfxB* sequence, second-step resistance mutants were isolated by plating dilutions of cells (ca. 5×10^9 CFU) on multiple plates containing increasing concentrations of NBTI 5463. Table 2 indicates the resistance rates, which were low. The resistant strains exhibited 8- to 16-fold increases in the MIC to NBTI 5463 compared to that of the parental first-step mutant strain (Table 3). Genomic DNA was isolated from several of the independently isolated resistant mutants, and the four topoisomerase genes (*gyrA*, *gyrB*, *parC*, and *parE*) were sequenced. Mutations were found exclusively in the *gyrA* gene (Table 3) and represented single base pair changes that affected the codon for aspartate 82 to generate glutamate, glycine, or asparagine. The asparagine substitution was the only change to significantly reduce fluoroquinolone susceptibility as well.

In order to test the effect of the GyrA mutations in the absence of the background NfxB expression, isolates with each of the three GyrA D82 mutations were transformed with the plasmid pMMB67 containing an intact *nfxB* gene from *P. aeruginosa* PAO1, as well as an empty vector control for each strain. Table S2 in the supplemental material contains the results, which illustrate that as expected, the GyrA D82 mutations by themselves raise the MIC level to NBTI, but except for D82N (which has a very modest effect), there is no impact of GyrA D82 mutations on fluoroquinolone resistance.

Although the MIC values of the second-step mutants were well beyond any clinically relevant level (128 to 256 μg/ml), attempts were made to select higher-level, next-step mutants. Beginning with a strain that had both a 4-bp NfxB deletion and an Asp82Glu GyrA change (AZ397), a few resistant mutants were obtained on LB plates with high levels of NBTI 5463. All had MIC values of >1,024 μg/ml. Sequencing of the four topoisomerase genes of four independent resistant isolates identified an identical additional change in GyrA of aspartate 87 to tyrosine in all four independent isolates (Table 4) evaluated. The strains also had increased ciprofloxacin and levofloxacin MIC values. High-level quinolone resistance has been previously reported with changes at the GyrA aspartate 87 locus (29).

We attempted to define the target potency of NBTI 5463 toward purified gyrase and TopoIV isolated from *P. aeruginosa*, but despite multiple attempts in protein production, we were unable to obtain either enzyme with satisfactory specific activity to allow for biochemical assays.

Resistance to NBTI in *E. coli*. As with *P. aeruginosa*, dense cultures (2×10^9 cells) of *E. coli* were spread on large-diameter LB agar plates containing multiples of the compound MIC (2×, 4×, 8×, 16×). This procedure was repeated multiple times, but no colonies grew on plates at 2× MIC or above. Five colonies were isolated during several attempts with 1× MIC (0.5 μg/ml) of NBTI 5463. However, when retested, they did not exhibit elevated MIC values compared with those for the parent strain. Genomic DNA was also isolated from these 1× MIC strains, and the topoisomerase genes were sequenced. None of these isolates had any mutations in the four type II topoisomerase subunits.

Because repeated efforts did not isolate resistant mutants of *E. coli*, the Wanner λ *red* recombineering system in plasmid pKD46 was employed to introduce changes at aspartate 82 of GyrA, analogous to the changes observed in resistant *P. aeruginosa* described above. The Asp82Glu and Asp82Gly mutations were successfully introduced into *E. coli*, but repeated attempts to introduce the Asp82Asn mutation were unsuccessful. Surprisingly, the introduction of these changes, which had a significant impact on NBTI resistance in *P. aeruginosa*, caused minimal change in susceptibility to either the NBTI compound or fluoroquinolones in *E. coli* (Table 5).

In order to explore further the effect of GyrA mutations on resistance to NBTI 5463 in *E. coli*, the GyrA Asp82Glu and Asp82Gly mutant strains were used for a second round of selection on LB plates against NBTI 5463. In this case, it was possible to select mutants at frequencies in the order of 10^{-8} to 10^{-9} with

TABLE 4 *P. aeruginosa* third-step high-level-resistance isolates

Strain[a]	MIC (μg/ml)[b]			Mutation[c]	
	NBTI 5463	Ciproflox.	Levoflox.	GyrA	NfxB
AZ397	128	1	2	D82E	(490–493) 4-bp deletion
AZ789	>1,024	8	8	D82E, D87Y	(490–493) 4-bp deletion
AZ790	>1,024	8	8	D82E, D87Y	(490–493) 4-bp deletion
AZ791	>1,024	8	8	D82E, D87Y	(490–493) 4-bp deletion
AZ792	>1,024	8	8	D82E, D87Y	(490–493) 4-bp deletion

[a] AZ789, -790, -791, and -792 were isolated at 8× MIC (1,024 μg/ml).
[b] Ciproflox., ciprofloxacin; Levoflox., levofloxacin.
[c] Mutations were not observed in *gyrB*, *parC*, or *parE* in any of these strains.

significantly decreased susceptibility (8 to 128-fold MIC increase) to NBTI 5463 (Table 5). Initially, these second-step mutants grew slowly on plates with NBTI 5463, taking 36 to 48 h to form small colonies, but immediately upon subsequent passage on LB plates with NBTI at the selection concentration grew at a normal rate. The initial slow growth may reflect a physiological shift or acquisition of additional compensatory mutations outside of the two topoisomerases. These second-step mutants had mutations in the ParC or ParE subunit of topoisomerase IV. The decreased susceptibility was confined to the NBTI compound, with no significant impact on the two fluoroquinolones tested.

The above results raised the question of whether the TopoIV mutations were solely responsible for the reduced susceptibility to NBTI 5463 or if it was also necessary to have the *gyrA* mutations in the strains' genetic background. To address this question, strains containing only the TopoIV mutations were constructed. Again, the DNA recombineering system was employed to first remove the Kan^r marker from *yfaL* and to subsequently place a Kan^r marker in the nonessential gene *ygiU*, adjacent to *parC* in the ASN484 and -489 strains. These were subsequently transduced via P1 phage into the parental *E. coli* W3110 background with selection for kanamycin resistance. The cotransduction of the TopoIV mutation was confirmed by DNA sequencing. As seen in Table 5, the presence of TopoIV subunit mutations alone was not sufficient to affect the susceptibility to NBTI 5463 or to the two fluoroquinolones tested.

With the observation that strains containing mutations in a single topoisomerase were not resistant to NBTI 5463, we next assessed whether the target-based mutations altered enzyme inhibition by NBTI 5463. Mutant *E. coli* GyrA and ParC proteins containing Asp82Gly and Asp79Gly, respectively, were expressed and purified. The mutant GyrA and ParC proteins were reconstituted with their parental GyrB or ParE partners, and enzyme activity was measured. Both reconstituted mutant topoisomerases exhibited specific activities comparable to those of their wild-type counterparts, and therefore, DNA-dependent ATPase assays were performed to measure 50% inhibitory concentrations (IC_{50}s) for NBTI 5463 (Table 6). While the IC_{50}s for the wild-type enzymes were within 2-fold of each other, the aspartate-to-glycine mutations in both enzymes resulted in large increases in the IC_{50}. The NBTI 5463 MIC values of *E. coli* strains containing only one of the mutant type II topoisomerases, with the other being wild type, were the same as the MIC of the wild-type strain (Table 6).

TABLE 5 MICs of *E. coli* strains with topoisomerase target mutations[a]

Strain[b]	MIC (μg/ml)			Mutation(s)		
	NBTI 5463	Ciproflox.	Levoflox.	GyrA	ParC	ParE
W3110	0.5	0.03	0.06			
AZ480	1	0.015	0.03	D82E		
AZ481	0.5	0.06	0.125	D82G		
AZ484	8	0.03	0.03	D82E	K277N, K278L	
AZ486	32	0.03	0.06	D82E		P439Q
AZ489	64	0.125	0.25	D82G	D79G	
AZ699	0.5	0.03	0.03		K277N, K278L	
AZ727	0.5	0.03	0.03		D79G	

[a] For all strains in the table, all four topoisomerase genes (*gyrA*, *gyrB*, *parC*, and *parE*) were sequenced.
[b] AZ484 was isolated at 2× MIC (2 μg/ml), AZ486 was isolated at 4× MIC (4 μg/ml), and AZ489 was isolated at 16× MIC (8 μg/ml).

DISCUSSION

The NBTI series of compounds, including NBTI 5463, was developed with the goal of improved Gram-negative pathogen coverage and minimizing the potential for hERG cardiac channel inhibition (30). Earlier compounds with chemical similarities to the NBTI series were focused primarily on Gram-positive antibacterial activities (10–16).

We previously reported on the properties of the Gram-negative-series compound NBTI 5463, including both *in vitro* and animal infection model studies (18). In the original report, the preliminary resistance studies found that in *P. aeruginosa*, a number of different mutations that affected the *nfxB* regulator of the MexCD, OprJ efflux pump system arose upon selection. In the present study, we extended the study of NBTI 5463 resistance in *P. aeruginosa* by employing one of the *nfxB* mutants to select second-step mutations. These next-level mutants arose at a low level, and employing increasing concentrations of compound led to a de-

TABLE 6 Inhibition of gyrase and topoisomerase IV by NBTI 5463

E. coli enzyme	GyrA/ParC status	IC_{50} (nM) of ATPase[a]	MIC (μg/ml) for *E. coli*[b]
Gyrase	GyrA wild type	5 ± 1	0.5
	GyrA D82G	>200	0.5
TopoIV	ParC wild type	2.6 ± 0.1	0.5
	ParC D79G	>200	0.5

[a] IC_{50}s (mean ± SD) for inhibition of the indicated enzyme by NBTI 5463.
[b] MIC versus strains W3110 for unaltered enzymes and strains AZ481 and AZ727 for the respective altered enzymes.

crease in the selection rate (Table 2). Sequencing of several mutants revealed that the resistance mutations were all found exclusively in the GyrA subunit, and the changes observed were isolated to a single locus, aspartate 82 of GyrA (Table 3). This residue is predicted to form a key compound binding interaction with the target, and mutations at the equivalent aspartate 83 in *S. aureus* have been reported to confer resistance to other NBTIs but not to alter fluoroquinolone susceptibility (10, 11). In *P. aeruginosa*, the aspartate 82 mutations all resulted in high-level NBTI resistance; however, fluoroquinolone susceptibility was unchanged in the Asp82Gly and Asp82Glu mutants but reduced 8-fold in the Asp82Asn mutant. As a possible explanation, given the proximity of aspartate 82 to threonine 83, which is known to influence fluoroquinolone susceptibility (29), it is conceivable that the Asp82Asn substitution alters the positioning of Thr83 enough to affect the water-metal ion bridge necessary for fluoroquinolone binding.

Employing these second-step gyrase mutants, a few isolates with extremely high-level resistance could also be selected (MIC > 1,024 μg/ml). In the clones isolated and tested, these mutations in response to NBTI 5463 were all found in this case to be in a second locus in GyrA, Asp87, that is associated with quinolone resistance, and indeed these isolates were cross resistant to the two fluoroquinolones tested. In *P. aeruginosa*, high-level fluoroquinolone resistance is associated with mutations in both gyrase and TopoIV, and mutations in gyrase appear to precede mutations in TopoIV (29, 31). The observed pattern for NBTI 5463, where in a GyrA mutant background, resistant mutations appeared again in GyrA, is an unusual finding. The appearance of the third-step mutant in a known fluoroquinolone resistance locus raises a concern about preexisting resistance to NBTI 5463. However, in a panel of 108 *P. aeruginosa* strains, NBTI 5463 displayed a MIC_{90} of 8 μg/ml for the 57 fluoroquinolone-resistant isolates tested, versus a MIC_{90} of 4 μg/ml for the 51 fluoroquinolone-susceptible strains (30). The isolates were not genotyped in that study, and future studies with genetically characterized strains that are susceptible and resistant to NBTI 5463 and fluoroquinolones are warranted.

A surprising finding was the repeated failure to raise any resistant mutants of *E. coli* against NBTI 5463. After several attempts, recombineering techniques were employed to introduce into *E. coli* the GyrA aspartate 82 mutations seen in *P. aeruginosa* by cotransduction with a selectable marker (Kan^r) in an adjacent gene. Neither the Asp82Gly nor Asp82Glu changes in *E. coli* GyrA led to an increase in the MIC against the NBTI compound or the two fluoroquinolones tested. However, employing these strains with gyrase mutations selected isolates on NBTI 5463 plates that did have increased MIC values for the NBTI, and mutations in several locations in *parC*, or in one case *parE*, were found in the resistant mutants. One of the *parC* mutants (AZ489) contained an Asp79Gly mutation that is analogous to the GyrA Asp82Gly mutation in its parent strain. As with gyrase, aspartate 79 in ParC is adjacent to a common fluoroquinolone resistance mutation location, serine 80 (32). It is noteworthy that the single Asp79Gly mutant constructed by transduction had no change in fluoroquinolone susceptibility.

One possibility was that *parC* mutations in the double mutant were solely responsible for the decreased susceptibility to NBTI 5463. To test this, two of the NBTI-resistant mutants (AZ484 and AZ489) were used to produce two strains with *parC* mutations only. The resulting strains were found to be susceptible to both NBTI 5463 and fluoroquinolones, similar to the GyrA-only mutants. We conclude that NBTI 5463 resistance requires mutations in two topoisomerase target genes in *E. coli*, since mutations in either gene alone did not affect susceptibility to NBTI 5463. This explains the initial failure to select mutants on plates with compound, since it would require the very unlikely creation of two simultaneous mutations in the gyrase and topoisomerase IV targets to select a resistant isolate.

The parental and mutant forms of the enzymes were compared for levels of enzyme inhibition. Two pieces of information emerged from this study (Table 6). One, the IC_{50}s for inhibition of gyrase and topoisomerase IV ATPase activities were very closely matched (within 2-fold) for the parent forms. Thus, the NBTI inhibitor is virtually equipotent in its *E. coli* target inhibition profile. Second, both mutant forms of the topoisomerase enzymes were resistant to NBTI 5463 inhibition. The maintained susceptibility seen in the single-target mutants demonstrates that NBTI 5463 is a dual-target inhibitor in *E. coli*. As a result of this property, the compound may encounter an *E. coli* strain with either a *gyrA* or *parC* single-target resistance mutation, but the equipotent nature of NBTI 5463 inhibition does not impact the MIC to permit this resistant mutant to survive and emerge.

The concept of dual targeting as an advantage for topoisomerase inhibitors has been recognized from the fluoroquinolone precedent (33–35). In Gram-positive pathogens, fluoroquinolones in general display greater potency toward TopoIV than gyrase, and as a result of this asymmetric target profile, earlier generations of quinolones readily selected for first-step ParC mutants. Recognizing that balanced, dual targeting should overcome this liability, subsequent evolution of the scaffold resulted in compounds with lower resistance rates that indeed possessed balanced *S. aureus* and *S. pneumoniae* gyrase-TopoIV profiles (36–39). In *P. aeruginosa* and *E. coli*, however, fluoroquinolones consistently select for gyrase first-step mutants and display greater target potency toward gyrase (29, 38).

For the NBTI class, knowledge is building on the target preferences in Gram-positive and Gram-negative pathogens and consequent ability to select for first-step target mutants. In one study, for example, NBTI analogs displayed equipotent target affinity for *Streptococcus pneumoniae* gyrase and TopoIV but an asymmetrical profile toward the *Staphylococcus aureus* enzymes, with greater affinity for *S. aureus* gyrase (40). Another group concluded that an asymmetrical target profile in *S. aureus* led to unacceptably high resistance frequencies (3×10^{-6} at 4× MIC) (41). This group directed compound optimization toward a balanced target profile in *S. aureus* and demonstrated that an analog with equipotent target affinities had a reduced spontaneous resistance mutation frequency (5×10^{-8} at 4× MIC).

Our work is the first to shed light on NBTI target preference and resistance potential in Gram-negative pathogens. *P. aeruginosa* and *E. coli* appear to follow different paths to resistance development. In our studies, changes in first-step mutants of *P. aeruginosa* appeared exclusively in an efflux pump regulator gene (42), and subsequent mutations appeared exclusively in two loci in gyrase only. The ability to select resistant strains carrying a mutation in gyrase suggests two possibilities: either NBTI 5463 has an unbalanced target profile in *P. aeruginosa* such that its TopoIV target affinity is significantly weaker than that for gyrase, or NBTI does inhibit topoisomerase IV potently in the mutant gyrase background but relies solely upon gyrase inhibition for antibacterial

activity, which translates to an increased MIC in the resistant mutants with GyrA mutations. With further investigation, NBTI 5463 may become a valuable tool compound for understanding *P. aeruginosa* topoisomerase inhibitor pharmacology.

The situation for NBTI 5463 in *E. coli* is clearly different from that in *P. aeruginosa*. In *E. coli*, the compound displayed exquisitely balanced target affinity such that inhibition of only one of the two targets translated to the same MIC value as if both targets were inhibited. This represents, to our knowledge, the first demonstration of such balanced, dual targeting of topoisomerases in a Gram-negative pathogen. As a result, in *E. coli*, NBTI 5463 exemplifies the ideal of requiring two simultaneous mutations for resistance development. Even though NBTI 5463 did not possess a preclinical safety profile to justify its progression (30), we believe that future NBTI molecules that realize the potential of this series to be successful Gram-negative antibacterial agents will be developed.

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