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

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Running title: Resistance to a novel topoisomerase inhibitor

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In a previous report (1), a novel bacterial type II topoisomerase inhibitor NBTI 5463 with activity against Gram negative pathogens was described. First-step resistance mutants 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 4-8 fold increases in the MIC over those seen in the parental strain with a first step *nfxB* efflux mutation. A third step mutant resulted in 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 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.
Introduction

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 (2-5). At the same time, attempts to identify fundamentally new targets and effective novel compounds to control multi-drug resistant (MDR) Gram negative infections have largely fallen short of their goals. Although many novel compounds have been reported in 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 (6-8), 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 (9,10), one strategy that has emerged is to identify new compounds that engage existing validated antimicrobial targets. There have been several reports of novel, non-quinolone 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 (11-18). We have recently reported on a chemical class of topoisomerase inhibitors with activity against several Gram negative multi-drug resistant pathogens; these compounds have been termed novel
bacterial type II topoisomerase inhibitors (NBTI) (1, 12). As previously reported, these compounds inhibit the two topoisomerase targets in the bacterial cell, gyrase and topoisomerase IV, in a manner distinct from 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 (1). 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 the susceptibility of 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 minimum inhibitory concentration (MIC) to the NBTI compound. Only after the introduction of mutations in both target enzymes was an increase in MIC observed.
Materials and Methods

Strains and media. Experiments to generate resistant strains for characterization were performed in *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 as AZ301 had a 4-bp deletion in the nfxB coding region (1), leading to an MIC increase against the NBTI compound over 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 onto 1×, 2×, 4×, and 8× the MIC of NBTI 5463 on large diameter (150 mm) plates. In addition, ten-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 hours 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 (1). DNA primers employed are listed in
Table S1. Dideoxy DNA sequencing was performed with an Applied Biosystems 3100 Series Genetic Analyzer.

Introduction of Asp82Glu and Asp82Gly in GyrA of E. coli. The wild type gyrA was cloned into pET-46 EK/LIC (EMD Millipore, Billerica, MA) to obtain pJT596. pAN125 and pAN126 were obtained by introducing missense mutations at Asp82Glu and Asp82Gly in pJT596 by site directed mutagenesis using the Quikchange kit from Agilent Technologies (Santa Clara, CA). The first 500 bp of the gyrA gene was amplified from pAN125 and pAN126. The PCR products were transformed into E. coli BW25113 (23,24). Two different isolates with mutations in Asp82Glu or Asp82Gly of 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 MIC change 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 non-essential genes were constructed.

Construction of the kanamycin-resistant strains for P1 phage co-transduction. P1 co-transduction relies on proximity of the genes. Hence the yfaL gene, located adjacent to the gyrA gene (Figure 1), was replaced by a kanamycin-resistant gene (KanR) (23) in strains containing Asp82Glu and Asp82Gly missense mutations in gyrA. P1 co-transduction was performed to move the tightly linked KanR gene and Asp82 mutations in gyrA into W3110 to obtain AZ480 and AZ481 (25, 26) by
selecting for kanamycin resistance. The proximity of the KanR marker to the gyrA
gene yielded greater than 95% co-transduction of the two genes with P1
transducing phage. The co-transduction 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 KanR
gene from the yfaL using PCP20 (23) for flippase-mediated excision from AZ484 and
AZ489. Subsequently, the ORF of ygiU (selected due to its proximity to the parC gene
– Figure 1) was replaced by the KanR gene for co-transduction of parC mutations
along with kanamycin resistance into the W3110 background. Thus, strains AZ687
and AZ717 were created, containing the KanR 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, Lys278Leu in parC) and AZ727 (Asp79Gly in parC). See Table 1
for strain genotypes.

Mutant topoisomerase production. The gene sequences encoding E. coli ParC (1-
752) Asp79Gly and E. coli GyrA (1-875) Asp82Gly were synthesized with an N-
terminal 6× His tag and a 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 (14, 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 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 (14, 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 (1). Using one of these strains, which contained a 4-base-pair deletion (490-493) in the 564 bp *nfxB* sequence, second-step resistance mutants were isolated by plating dilutions of cells (ca. 5 $\times$ 10$^9$ CFU) on multiple plates containing increasing concentrations of NBTI 5463. Table 2A indicates the resistance rates, which were low. The resistant strains exhibited 8-16 fold increases in MIC to NBTI 5463 compared to the parental first-step mutant strain (Table 2B). 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 2B), and represented single base pair changes that affected the
codon for aspartate 82 to generate glutamate, glycine or asparagine. The
asparaginase 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
contains the results, which illustrate that as expected, the GyrA D82 mutations by
themselves raise the MIC level to NBTI, but except for the 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 (128-256 μg/ml), attempts were made to select higher level, next-step
mutants. Beginning with a strain that had both a four base pair 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 >1024 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 3) 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 towards 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.

**NBTI Resistance in *E. coli***. As with *P. aeruginosa*, dense cultures (2 x 10⁹ cells) of *E. coli* were spread onto 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 the parent strain. Genomic DNA was also isolated from these 1× MIC strains and the topoisomerase genes sequenced. None of these isolates had any mutations in the four type II topoisomerase subunits.

Because repeated efforts could not isolate resistant mutants in *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 4).

In order to explore further the effect of GyrA mutations on NBTI 5463 resistance 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 significantly decreased susceptibility (8 to 128-fold MIC increase) to NBTI 5463 (Table 4). Initially, these second-step mutants grew slowly on plates with NBTI 5463, taking 36-48 hours 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 subunits 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 whether the TopoIV mutations were solely responsible for the reduced susceptibility to NBTI 5463, or was it 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 KanR marker from *yfaL* and to subsequently place a KanR marker into the non-essential 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 4, 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 were expressed and purified containing Asp82Gly and Asp79Gly, respectively. 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 comparable specific activity to their wild-type counterparts and therefore DNA-dependent ATPase assays were performed to measure IC₅₀ values for NBTI 5463 (Table 5). While the IC₅₀ values for the wild-type enzymes were within two-fold of each other, the aspartate to glycine mutations in both enzymes resulted in large increases in the IC₅₀. 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 5).
Discussion

The NBTI series of compounds including NBTI 5463 were 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 (11-17).

We previously reported on the properties of the Gram negative series compound NBTI 5463, including both in vitro and animal infection model studies (1). 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 decrease in the selection rate (Table 2A). 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 2B). This residue is predicted to form a key compound binding interaction to the target and mutations at the equivalent aspartate 83 in *S. aureus* have been reported to confer resistance to other NBTIs but not alter fluoroquinolone susceptibility (11, 12). 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 that 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 >1024 μg/ml). In the clones isolated and tested, these mutations 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 pre-existing resistance to NBTI 5463. However, in a panel of 108 P. aeruginosa strains, NBTI 5463 displayed a MIC₉₀ of 8 μg/mL in the 57 fluoroquinolone-resistant isolates tested versus a MIC₉₀ of 4 μg/mL in 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 in *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 co-transduction with a selectable marker (KanR) in an adjacent gene. Neither the Asp82Gly nor Asp82Glu changes in *E. coli* GyrA led to an increase in 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 to 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 a 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). Noteworthy is 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, 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 NBTI 5463 susceptibility. This explains the initial failure to select mutants on plates with compound, as 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 5). One, the
IC₅₀ values 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 precedence (33-35). In Gram-positive
pathogens, fluoroquinolones in general display greater potency towards TopoIV
than gyrase, and as a result of this asymmetric target profile, earlier generations of
quinolones readily selected for first-step mutants in ParC. 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.*
*P. aeruginosa* and *E. coli*, however, fluoroquinolones consistently select for gyrase first-step mutants and display greater target potency towards 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 report for example, NBTI analogs displayed equipotent target affinity for *Streptococcus pneumoniae* gyrase and TopoIV, but an asymmetrical profile towards 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 towards 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, first step mutants in *P. aeruginosa* appeared exclusively in an efflux pump regulator gene (42), and subsequent mutants appeared exclusively in two loci in gyrase only. The ability to select resistance mutants 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 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 *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 will be developed that realize the potential of this series to be successful Gram-negative antibacterial agents.
Acknowledgements

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Table 1. Strain details

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<th>Genotype</th>
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<td>AZ301</td>
<td>4 bp deletion from 490bp to 493 bp in <em>nfxB</em>.</td>
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<td>AZ391</td>
<td>D82E in GyrA, 4 bp deletion from 490bp to 493 bp in <em>nfxB</em>.</td>
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<td>AZ392</td>
<td>D82E in GyrA, 4 bp deletion from 490bp to 493 bp in <em>nfxB</em>.</td>
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<tr>
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<td>D82E in GyrA, 4 bp deletion from 490bp to 493 bp in <em>nfxB</em>.</td>
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<td>D82N in GyrA, 4 bp deletion from 490bp to 493 bp in <em>nfxB</em>.</td>
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<td>AZ480</td>
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<td>D82G in GyrA, yfaL is replaced by kanR.</td>
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<td>D82E in GyrA, K277N and K278L in ParC, yfaL is replaced by kanR.</td>
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<td>D82E in GyrA, P439Q in ParE, yfaL is replaced by kanR.</td>
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<td>AZ489</td>
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<td>pKD46 (23) is present.</td>
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<td>K277N and K278L in ParC, markerless deletion of yfaL, ygiU is replaced by kanR.</td>
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</tbody>
</table>
AZ704  D82E in GyrA, D79G in ParC, markerless deletion of yfaL.
AZ711  
AZ717  
AZ727  D79G in ParC, markerless deletion of yfaL, ygiU is replaced by kanR.
Table 2A.

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

<table>
<thead>
<tr>
<th>Concentration, ×MIC (8 μg/ml)</th>
<th>Mutation rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1×</td>
<td>&gt; 5.4 × 10⁻⁹</td>
</tr>
<tr>
<td>2×</td>
<td>1.1 × 10⁻⁹</td>
</tr>
<tr>
<td>4×</td>
<td>5.0 × 10⁻¹⁰</td>
</tr>
<tr>
<td>8×</td>
<td>&lt; 5.4 × 10⁻¹⁰</td>
</tr>
</tbody>
</table>
### Table 2B.

MIC values and sequence data for gyrase A second step *P. aeruginosa* mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>GyrA designation</th>
<th>GyrA mutation</th>
<th>MIC (µg/ml)</th>
<th>NBTI 5463</th>
<th>Ciprofloxacin</th>
<th>Levofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA01</td>
<td>-</td>
<td>0.5</td>
<td>0.13</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ301 (nfxB Δ490–493)</td>
<td>-</td>
<td>16</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ391*</td>
<td>D82E</td>
<td>256</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ392</td>
<td>D82E</td>
<td>256</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ393</td>
<td>D82E</td>
<td>256</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ394</td>
<td>D82N</td>
<td>256</td>
<td>8</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ395</td>
<td>D82N</td>
<td>256</td>
<td>8</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ396</td>
<td>D82G</td>
<td>128</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ397</td>
<td>D82E</td>
<td>128</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ398</td>
<td>D82N</td>
<td>256</td>
<td>8</td>
<td>16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*AZ391, 392, 393, 394 were isolated at 2× MIC (32 µg/ml). AZ395, 396, 397, 398 were isolated at 4× MIC (64 µg/ml).*
Table 3. *P. aeruginosa* third step high level resistance isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)</th>
<th>Mutations&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ397</td>
<td>128</td>
<td>D82E (490-493) 4 bp deletion</td>
</tr>
<tr>
<td>AZ789&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1024</td>
<td>D82E, D87Y (490-493) 4 bp deletion</td>
</tr>
<tr>
<td>AZ790</td>
<td>&gt;1024</td>
<td>D82E, D87Y (490-493) 4 bp deletion</td>
</tr>
<tr>
<td>AZ791</td>
<td>&gt;1024</td>
<td>D82E, D87Y (490-493) 4 bp deletion</td>
</tr>
<tr>
<td>AZ792</td>
<td>&gt;1024</td>
<td>D82E, D87Y (490-493) 4 bp deletion</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mutations were not observed in *gyrB, parC*, or *parE* in any of these strains

<sup>b</sup>AZ789, 790, 791, 792 were isolated at 8× MIC (1024 µg/ml)
Table 4. MIC values (μg/ml) of *E. coli* strains with topoisomerase target mutations

<table>
<thead>
<tr>
<th>Strain</th>
<th>NBTI 5463</th>
<th>Ciproflox.</th>
<th>Levoflox.</th>
<th>GyrA</th>
<th>ParC</th>
<th>ParE</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>0.5</td>
<td>0.03</td>
<td>0.06</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AZ480</td>
<td>1</td>
<td>0.015</td>
<td>0.03</td>
<td>D82E</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AZ481</td>
<td>0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>D82G</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AZ484b</td>
<td>8</td>
<td>0.03</td>
<td>0.03</td>
<td>D82E</td>
<td>K277N, K278L</td>
<td>-</td>
</tr>
<tr>
<td>AZ486</td>
<td>32</td>
<td>0.03</td>
<td>0.06</td>
<td>D82E</td>
<td>-</td>
<td>P439Q</td>
</tr>
<tr>
<td>AZ489</td>
<td>64</td>
<td>0.125</td>
<td>0.25</td>
<td>D82G</td>
<td>D79G</td>
<td>-</td>
</tr>
<tr>
<td>AZ699</td>
<td>0.5</td>
<td>0.03</td>
<td>0.03</td>
<td>-</td>
<td>K277N, K278L</td>
<td>-</td>
</tr>
<tr>
<td>AZ727</td>
<td>0.5</td>
<td>0.03</td>
<td>0.03</td>
<td>-</td>
<td>D79G</td>
<td>-</td>
</tr>
</tbody>
</table>

For all strains in the table, all four topoisomerase genes (*gyrA*, *gyrB*, *parC* and *parE*) were sequenced.

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).
Table 5.

Inhibition of gyrase and topoisomerase IV by NBTI 5463

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>GyrA/ParC status</th>
<th>ATP-ase IC$_{50}$ (nM)</th>
<th>MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli GyrA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GyrA wild-type</td>
<td>GyrA wild-type</td>
<td>5 ± 1</td>
<td>0.5</td>
</tr>
<tr>
<td>GyrA D82G</td>
<td>GyrA D82G</td>
<td>&gt;200</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>E. coli TopoIV</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ParC wild-type</td>
<td>ParC wild-type</td>
<td>2.6 ± 0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>ParC D79G</td>
<td>ParC D79G</td>
<td>&gt;200</td>
<td>0.5</td>
</tr>
</tbody>
</table>

$^a$IC$_{50}$ values for inhibition of the indicated enzyme by NBTI 5463.

$^b$MIC vs. strains W3110 for unaltered enzymes, and AZ481 and AZ727 for the respective altered enzymes.
Figure 1. Closely linked genes are highly co-transduced by P1 phage generalized transduction. The kanamycin resistance genes were placed in yfaL and ygiiL. (Top) Gene context for E. coli gyrA and yfaL, spanning approximately 6 Kb. (Bottom) Gene context for E. coli parC and ygiiL, spanning roughly 4 Kb. P1 co-transduction of these closely linked gene exceeds 95%.