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NBTI 5463 Is a Novel Bacterial Type II Topoisomerase Inhibitor with Activity against Gram-Negative Bacteria and *In Vivo* Efficacy

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The need for new antibiotics that address serious Gram-negative infections is well recognized. Our efforts with a series of novel bacterial type II topoisomerase inhibitors (NBTIs) led to the discovery of NBTI 5463, an agent with improved activity over other NBTIs against Gram-negative bacteria, in particular against *Pseudomonas aeruginosa* (F. Reck, D. E. Ehmann, T. J. Dougherty, J. V. Newman, S. Hopkins, G. Stone, N. Agrawal, P. Ciaccio, J. McNulty, H. Barthlow, J. O'Donnell, K. Goteti, J. Breen, J. Comita-Prevoir, M. Cornebise, M. Cronin, C. J. Eyermann, B. Geng, G. R. Carr, L. Pandarinathan, X. Tang, A. Cottone, L. Zhao, N. Bezdenejnih-Snyder, submitted for publication). In the present work, NBTI 5463 demonstrated promising activity against a broad range of Gram-negative pathogens. In contrast to fluoroquinolones, the compound did not form a double-strand DNA cleavable complex with *Escherichia coli* DNA gyrase and DNA, but it was a potent inhibitor of both DNA gyrase and *E. coli* topoisomerase IV catalytic activities. In studies with *P. aeruginosa*, NBTI 5463 was bactericidal. Resistant mutants arose at a low rate, and the mutations were found exclusively in the *nfxB* gene, a regulator of the MexCD-OprJ efflux system. Levofloxacin-selected resistance mutations in GyrA did not result in decreased susceptibility to NBTI 5463. Animal infection studies demonstrated that NBTI 5463 was efficacious in mouse models of lung, thigh, and ascending urinary tract infections.

Gram-negative pathogens have become an increased focus for antibiotic development with the continued erosion of the efficacy of current therapies (1). Current options to treat Gram-negative infections are becoming alarmingly limited due to the organisms' abilities to evade existing antibiotic classes by employing a broad array of resistance mechanisms (2). Multidrug-resistant (MDR) Gram-negative bacteria represent important nosocomial pathogens and are responsible for a significant proportion of infections in patients in hospital and intensive care unit (ICU) settings (3). It is clear that additional agents effective against Gram-negative organisms, in particular *Pseudomonas aeruginosa*, are needed (4).

The bacterial topoisomerases have proven to be very effective targets for the fluoroquinolone class of antibiotics (5, 6). Bacterial type II topoisomerases are enzymes that mediate transient double-strand DNA breaks and participate in DNA replication and decatenation reactions (7). DNA gyrase can introduce negative supercoils and controls the level of supercoiling in the bacterial chromosomal DNA (8, 9). Topoisomerase IV is most efficient in decatenating activity, and participates in daughter chromosome separation (10, 11). DNA gyrase is a heterotetramer composed of two copies of each of two protein subunits, GyrA and GyrB (12). Topoisomerase IV is similarly a tetramer of two homodimeric subunits, designated ParC and ParE (13). The fluoroquinolone antibiotics inhibit DNA replication by forming complexes of the drug with DNA bound to the topoisomerase enzyme. This complex acts as a poison for DNA replication, blocking the progression of the replication fork and subsequently inducing the formation of double-strand breaks in the chromosome (14). Despite clinical success, the utility of the fluoroquinolones has eroded over time with use, due primarily to point mutations in the two target enzymes, bacterial gyrase and topoisomerase IV, as well as drug efflux pump mechanisms (15).

In this report, we describe the properties of a novel bacterial type II topoisomerase inhibitor (NBTI), NBTI 5463. Mechanistic studies with NBTIs have revealed that members of this class are similar to the fluoroquinolones in that they bind to the DNA-bound form of gyrase and topoisomerase IV; however, structural studies have revealed a binding site distinct from that of fluoroquinolones (16, 17). Our earlier effort in this series of compounds, as well as recent work from other investigators, was focused on optimizing potency and safety, with an emphasis on Gram-positive pathogens (18–22). The discovery of NBTI 5463, whose spectrum of activity includes *P. aeruginosa* and is not affected by the target-based point mutations associated with resistance to fluoroquinolones, represents an advance with this emerging class of agents.

MATERIALS AND METHODS

Strains and media. Clinical isolates of Gram-negative bacteria in the AstraZeneca culture collection were used for initial MIC determinations. These included multiple strains of *P. aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, *Enterobacter* spp., *Proteus* spp., and *Citrobacter* spp. *Pseudomonas aeruginosa* strain PAO1 was employed for mutational and time-kill studies. Susceptibility testing was performed in cation-adjusted Mueller-Hinton broth according to Clinical

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and Laboratory Standards Institute (CLSI) guidelines (23). The appropriate quality control strains (*E. coli* ATCC 25922 or *P. aeruginosa* ATCC 27853) were used in each susceptibility experiment. The medium for routine growth, time-kill studies, and resistance studies was LB broth or solid LB agar medium. Antibiotics and NBTI 5463 were dissolved at high concentrations in dimethyl sulfoxide (DMSO) and diluted.

Killing kinetics. Cells were grown overnight in LB broth at 37°C with aeration. The next morning, the cells were diluted to approximately 10^6 CFU/ml in 15 ml of LB, followed by the addition of test compound at the indicated concentrations, along with an untreated control. At timed intervals, 0.1-ml samples were removed and diluted in LB broth in a 10-fold series, and 0.1-ml samples from the dilutions spread on an LB agar plate. The number of CFU was counted after 24 h of incubation at 37°C.

Resistant mutant selection. *P. aeruginosa* PAO1 was grown overnight at 37°C in LB broth. The next morning, 0.1-ml samples were spread onto agar plates containing concentrations of compound from 2-fold to 32-fold of the broth microdilution MIC. The plates were incubated for 48 h, and the number of colonies that formed was determined. In addition, a sample of the overnight culture was diluted in 10-fold steps and plated on LB agar to determine the number of CFU/ml present. Several of the resulting colonies were selected and retested for changes in susceptibility profiles, and potential candidate resistance genes were amplified by PCR.

Gene amplification and sequencing. The genes for the topoisomerase subunits and several efflux pumps were amplified from *P. aeruginosa* genomic DNA. Selective amplification of high-GC-content genomic DNA from *P. aeruginosa* required the presence of DMSO to obtain full-length amplification of the larger topoisomerase gene subunits. PCR was carried out in 50-μl volumes with 25 μl of 2× Roche high-fidelity master mix, forward and reverse primers (10 pmol each), 4 μl DMSO (molecular biology grade from Sigma), 100 ng of template DNA, and nuclease-free water. Amplification was performed at 94°C for 5 min., followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 3 min. A final 72°C extension step was held for 7 min, and then the reaction mixture was held at 4°C until analyzed. The DNA primers employed and typical amplification of target genes are illustrated in Table S1 and Fig. S1 in the supplemental material, respectively. Dideoxy DNA sequencing was performed with a Life Sciences 3100 series Genetic Analyzer.

RT-PCR. The parent PAO1 and the *nfxB* mutants were grown overnight in LB at 37°C with shaking at 200 rpm. The strains were then subcultured (1:100) into 5 ml LB medium, grown at 37°C with shaking at 200 rpm, and then harvested at an A_{600} of 1.2 to 1.5. Total RNA isolation, primer synthesis, synthesis of cDNA, and real-time PCR (RT-PCR) analysis were performed as described by Dumas et al. (24). RT-PCR of the housekeeping gene *rpsL* was employed as an internal quantitation control. The instrument used to quantify the cDNA was a Chromo 4 system CFB-3240 (Bio-Rad, CA), and the software used was Opticon Monitor version 3.1. The DNA primers employed are listed in Table S2 in the supplemental material.

Topoisomerase activity assays. Measurement of the formation of a cleavable complex with *E. coli* gyrase was performed as described previously (16). Measurement of the inhibition of the catalytic activity of *E. coli* gyrase was performed using a fluorescence polarization assay as described previously (25). Measurement of the inhibition of the catalytic activity of *E. coli* topoisomerase IV was performed using an ATPase assay as described previously (18).

***P. aeruginosa* and *E. coli* neutropenic thigh infection model.** All studies were performed under approved IACUC protocols and conform to Office of Laboratory Animal Welfare (OLAW) standards. NBTI 5463 was studied in a neutropenic mouse thigh infection model. Briefly, CD-1 mice were rendered neutropenic by injecting cyclophosphamide (Sigma-Aldrich, St. Louis, MO) intraperitoneally at 4 days (150 mg/kg of body weight) and 1 day (100 mg/kg) before experimental infection. Mice were infected with a mid-log-phase culture of either *P. aeruginosa* PAO1, *E. coli* ATCC 25922, or *E. coli* ARC1710 (a clinical isolate from the AstraZeneca culture collection) to achieve a target inoculum of 5×10^5 CFU. Groups of five animals each received an intraperitoneal injection of between 5 and 200 mg/kg/day of drug prepared in 5% dextrose with lactic acid (pH 5.0) on a twice-a-day (b.i.d.), every-12-h (q12) regimen starting 2 h after infection. An additional group of five mice received vehicle alone. Efficacy was determined 24 h after the start of treatment. Thighs were removed, weighed, and homogenized, and aliquots were serially diluted and plated onto tryptic soy agar plates and incubated at 37°C overnight for determination of CFU/g thigh.

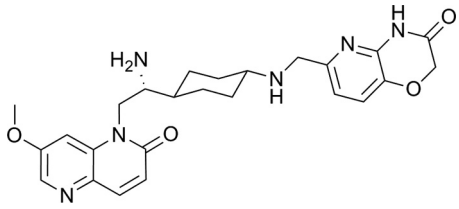

FIG 1 Structure of NBTI 5463.

***P. aeruginosa* neutropenic lung infection model.** Mice were rendered neutropenic as described above and infected with a mid-log-phase culture of *P. aeruginosa* PAO1 to achieve a target inoculum of 1×10^6 CFU via direct intratracheal instillation. Groups of 10 animals each received an intraperitoneal injection of between 5 and 200 mg/kg/day prepared in 5% dextrose with lactic acid (pH 5.0) on a b.i.d., q12 regimen starting 2 h after infection. An additional group of 10 mice received vehicle alone. Efficacy was determined 24 h after the start of treatment. Lungs were removed, homogenized, and serially diluted, and aliquots were plated onto *Pseudomonas* isolation agar plates and incubated at 37°C for 24 h prior to determination of CFU/lung.

***P. aeruginosa* and *E. coli* immunocompetent ascending urinary tract infection model.** The strains employed were clinical isolates *P. aeruginosa* VL-098 (MIC for NBTI 5463 = 1 μg/ml) and *E. coli* VL-229 (MIC for NBTI 5463 = 1 μg/ml). Female BALB/c mice weighing 18 to 20 g were placed on a regimen of 5% glucose–water 5 days prior to infection. Under anesthesia, mice were injected intraurethrally with 50 μl saline containing approximately 8×10^7 CFU of *P. aeruginosa* VL-098 or *E. coli* VL-229. At 24 h postinfection, groups of 10 mice were treated with various doses of gentamicin injected subcutaneously or NBTI 5463 injected intraperitoneally at 10, 30, or 100 mg/kg once daily for 3 days. At 24 h after the last treatment, the animals were euthanized. Kidneys and bladders were aseptically removed, weighed, homogenized, serially diluted, and plated. CFU per gram of kidney and bladder were calculated. Statistical significance of the differences between groups was assessed using the Mann-Whitney one-tailed U test (26). *P* values of <0.05 were considered significant.

PK/PD analysis. The CFU determinations resulting from the thigh and lung models were assessed for outliers using the interquartile method, and statistical outliers were removed prior to pharmacokinetic/pharmacodynamic (PK/PD) analysis. Pharmacokinetic parameters were assessed as described previously (F. Reck et al., submitted for publication). The exposure-response plots were modeled according to an E_{max} model, and PK/PD magnitudes for the efficacy endpoints were calculated using WinNonlin v5.2. The magnitudes associated with the free plasma area under the curve (AUC)/MIC ratio were determined based upon previous results which indicated that the AUC/MIC ratio was the PK/PD index best associated with efficacy (27). Free plasma values were calculated using a mouse protein binding value of 21.5%. The MIC values for *E. coli* strains ATCC 25922 and ARC1710 were 2 μg/ml. The MIC value for *P. aeruginosa* strain PAO1 was 0.5 μg/ml.

RESULTS

MIC determinations against a range of Gram-negative pathogens. NBTI compounds optimized for activity against Gram-negative pathogens were derived from a prior program on N-linked aminopiperidines focused on Gram-positive pathogens (18). The present compound series is exemplified by NBTI 5463 (Fig. 1).

TABLE 1 MIC distributions for NBTI 5463 against key Gram-negative bacterial pathogens

Bacterial species (no. of strains)[a]	Agent	MIC[b] (μg/ml)		
		MIC_{50}	MIC_{90}	Range
Escherichia coli (20)	NBTI 5463	1	2	0.5–4
	Levofloxacin	≤0.06	16	≤0.06–32
	Cefepime	≤0.06	>64	≤0.06–>64
	Gentamicin	1	2	0.05–>64
Klebsiella pneumoniae (20)	NBTI 5463	4	16	0.5–>64
	Levofloxacin	0.5	64	≤0.06–>64
	Cefepime	0.25	>64	<0.06–>64
	Gentamicin	0.5	64	0.25–>64
Proteus spp. (30)	NBTI 5463	2	8	0.125–>64
	Levofloxacin	0.06	2	0.015–4
	Ceftazidime	0.06	0.25	0.03–32
	Amikacin	4	8	1–16
Enterobacter spp. (30)	NBTI 5463	4	16	0.5–32
	Levofloxacin	0.125	4	0.03–4
	Ceftazidime	8	32	0.25–32
	Amikacin	2	8	2–64
Citrobacter spp. (30)	NBTI 5463	0.5	4	0.06–8
	Levofloxacin	0.03	4	0.008–4
	Ceftazidime	0.5	32	0.125–32
	Amikacin	2	8	0.5–>64
Pseudomonas aeruginosa (21)	NBTI 5463	0.5	2	0.125–4
	Levofloxacin	1	32	0.125–32
	Cefepime	8	16	1–64
	Gentamicin	4	>64	1–>64
Stenotrophomonas maltophilia (20)	NBTI 5463	4	8	0.25–32
	Levofloxacin	0.5	2	0.125–8
	Cefepime	64	>64	32–>64
	Gentamicin	64	>64	4–>64
Acinetobacter baumannii (20)	NBTI 5463	8	64	0.125–>64
	Levofloxacin	8	32	<0.06–>64
	Cefepime	16	>64	<0.06–>64
	Gentamicin	64	>64	1–>64

[a] Bacterial strains from the AstraZeneca internal collection of clinical isolates.
[b] MICs determined in Mueller-Hinton broth according to CLSI methods.

This compound exhibited improved activity against Gram-negative bacteria, low plasma protein binding (73% free fraction), low hERG channel inhibition (50% inhibitory concentration [IC_{50}], >333 μM), and high solubility (>1,000 μM) (Reck et al., submitted for publication). Preliminary MIC testing with a broad set of Gram-negative clinical isolates in the AstraZeneca culture collection indicated potent activity against this panel of isolates and strains (Table 1). From the *P. aeruginosa* isolates in this panel, a subset that exhibited multidrug resistance (MDR) phenotypes is excerpted in Table 2. NBTI 5463 was found to have potent activity against these problematic isolates, whereas comparator compounds in different antibiotic classes had increased MIC values relative to those for the susceptible ATCC 27853 comparator strain.

Time-kill kinetics. In time-kill studies, NBTI 5463 demonstrated concentration-dependent bactericidal behavior over an 8-h period against *P. aeruginosa* PAO1 (Fig. 2). Also evident was regrowth at 24 h of exposure at concentrations from 1× to 8× the MIC value. For comparison, levofloxacin was profiled in a parallel time-kill study. Compared to NBTI 5463, levofloxacin displayed more rapid cidality, and although regrowth with levofloxacin was evident at 4× MIC, at 8× MIC, no regrowth was observed. This may reflect the fact that levofloxacin resistance mutations obtained in the strains at 2× to 4× MIC were all found to be in gyrase and hence were not efflux (*nfxB*)-based first-step resistance mutations (see Table 5). The higher rate of killing by levofloxacin may be a reflection of the difference in the detailed mechanism of gyrase inhibition by the NBTI compound (e.g., the lack of double-

TABLE 2 MICs of *Pseudomonas aeruginosa* MDR strains

Strain[a]	MIC (μg/ml)			
	NBTI 5463	Levofloxacin	Cefepime	Gentamicin
ATCC 27853	0.5	1	2	2
Pae2655	0.125	32	16	8
Pae2657	0.5	16	32	>64
Pae2661	0.25	16	8	>64
Pae2662	0.25	16	64	8
Pae2759	0.5	32	16	>64

[a] Strains from the AstraZeneca internal collection of clinical isolates.

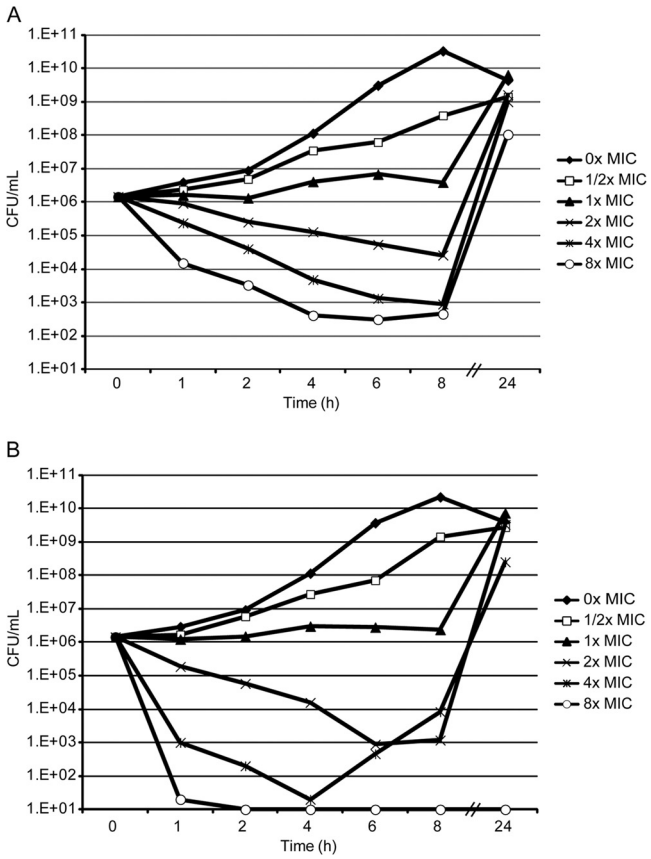

FIG 2 (A) Time-kill kinetics of *Pseudomonas aeruginosa* PAO1 exposed to NBTI 5463. Concentrations range from 0 up to 8× the MIC (1× MIC = 0.5 μg/ml). (B) Time-kill kinetics of *Pseudomonas aeruginosa* PAO1 exposed to levofloxacin.

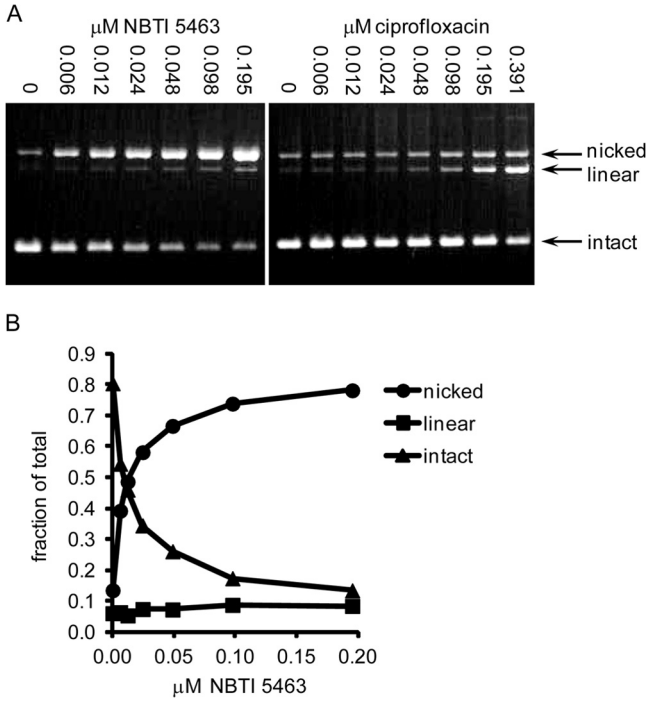

FIG 3 Cleavable complex assay with *E. coli* gyrase. (A) Formation of DNA gyrase cleavable complexes with DNA in the presence of increasing concentrations of NBTI 5463 and ciprofloxacin. The agarose gel migration locations of nicked, cleaved, and intact DNA are labeled. (B) Replot of relative percentages of nicked, cleaved, and intact DNA from the NBTI 5463 titration.

strand cleavage; see below). Colonies of *P. aeruginosa* from exposure to 4× the MIC of NBTI 5463 were subjected to gene amplification and analysis (see "Resistance selection and analysis" below) to determine if the regrowth was due to selection of resistant mutants over the time period of exposure.

Topoisomerase inhibition. In contrast to the situation observed with increasing concentrations of fluoroquinolones, the NBTI compounds do not result in formation of a double-strand DNA cleavable complex (22, 28, 29) (Fig. 3A). This indicates that the inhibition of topoisomerase activity by NBTI compounds does not happen at the stage in which both DNA strands have been broken and covalently bound to gyrase. Rather, NBTI 5463 engages the topoisomerase target while it is bound to DNA but prior to double-strand breakage, a mode of inhibition for NBTIs suggested by the X-ray structure of a ternary *Staphylococcus aureus* gyrase-DNA-NBTI complex (17) as well as by results from biochemical competition experiments with a fluoroquinolone (16). Also consistent with this binding mode is the observation of release of nicked DNA upon treatment with NBTI 5463 (Fig. 3B). Gyrase binds DNA in the absence of ATP, and it has been shown that with ATP bound the reaction equilibrium lies toward the direction of double-stranded DNA cleavage (7). The binding of NBTI 5463 to the gyrase-DNA complex prevents formation of double-strand breaks but may allow the transient formation of single-strand breaks, which are released and visualized in the cleavage complex assay.

Further differentiation of NBTI 5463 from the fluoroquinolone class can be seen in the comparison of enzyme turnover IC_{50}s to MIC values. As shown in Table 3, the IC_{50}s for ciprofloxacin against its topoisomerase target are significantly higher than its MIC, especially in the *tolC* background. For fluoroquinolones this phenomenon is explained by their action as topoisomerase poisons that promote the formation of toxic lesions in DNA at very low intracellular drug concentrations (30). Compared to ciprofloxacin, NBTI 5463 was found to be 10- to 100-fold more potent an inhibitor of turnover of *E. coli* gyrase and topoisomerase IV, respectively. The IC_{50}s for NBTI 5463 are similar in magnitude to its MIC in the *tolC* background. These results, taken with the lack of double-strand DNA cleavable complex formation, are consistent with NBTI 5463 acting as a catalytic inhibitor of topoisomerases.

TABLE 3 Topoisomerase activity inhibition assays

Agent	IC_{50}, μM		MIC, μM[a]	
	E. coli gyrase supercoiling	*E. coli* topoisomerase IV ATPase	*E. coli* ARC523 (W3110)	*E. coli tolC* ARC524 (W3110)
Ciprofloxacin	0.3	6	0.06	<0.005
NBTI 5463	0.02	0.003	0.5	0.05

[a] MICs converted to μM.

Resistance selection and analysis. Resistance frequencies of *P. aeruginosa* were determined by spreading cells from a late-logarithmic-phase liquid culture onto LB agar plates that had increasing concentrations of NBTI 5463 (Table 4). The resistance rates at low MIC multiples were in the range of 10^{-8}, and they decreased with increasing compound concentration. Four independent colonies from different plates were selected for more extensive MIC studies, and the results of those studies are shown in Table 5. Decreased susceptibility was observed for the NBTI compound, as well as for two fluoroquinolones and both cefepime and tetracycline. In contrast, meropenem exhibited increased susceptibility. The increase in resistance for the "fourth-generation" cephalosporin cefepime and tetracycline, accompanied by an increase in meropenem sensitivity, also strongly suggested that the resistance mechanism was not through topoisomerase target alteration but might be through modified uptake or efflux.

To determine the nature of the resistance, several of the *Pseudomonas* NBTI 5463-resistant mutants were grown in liquid culture in the presence of the compound, the genomic DNA was isolated, and selected genes were amplified and sequenced. Primers (see Table S1 and Fig. S1 in the supplemental material) for the four topoisomerase subunit genes (*gyrA*, *gyrB*, *parC*, and *parE*) were employed, as well as primers for multiple efflux pump regulator genes (*mvrA*, *nfxB*, *mexR*, *mexS*, *mexT*, and *mexZ*). In the

TABLE 4 *Pseudomonas aeruginosa* resistance mutation rates for NBTI 5463[a]

Concn, μg/ml (× MIC)	Resistance rate
1 (2)	1.8×10^{-7}
2 (4)	5×10^{-8}
4 (8)	3×10^{-8}
8 (16)	1×10^{-8}
16 (32)	Below detection limit

[a] *P. aeruginosa* PAO1 was grown to 7×10^9 CFU/ml, and a total of 0.3 ml was spread on an LB plate with the indicated compound concentration. The MIC for this strain was 0.5 μg/ml. Colonies were counted at 48 h of incubation.

TABLE 5 *Pseudomonas aeruginosa* mutant isolate MICs

Strain[a]	Mutation(s)	MIC (μg/ml)[b]					
		NBTI 5463	LVX	CIP	FEP	TET	MEM
PAO1 parent		0.5	0.5	0.12	1	8	4
AZ301	*nfxB* 4-bp deletion at bp 490–493	8	4	1	4	32	0.5
AZ302	*nfxB* stop codon at bp 100	8	4	1	8	32	0.5
AZ303	*nfxB* multiple mutations from bp 341 onwards (including stop codon)	4	1	0.5	4	16	0.25
AZ304	*nfxB* deletion at bp 42–267	8	4	1	4	32	0.5
AZ-LV-1	GyrA T83I	<0.063	4	1	1	8	4
AZ-LV-2	GyrA T83I	<0.063	8	2	1	16	8
AZ-LV-3	GyrB E468D	0.25	4	2	2	8	4

[a] The AZ-LV mutants were three independent mutants selected on 4× MIC of levofloxacin. Several additional levofloxacin-resistant strains were also characterized; all had gyrase mutations.
[b] Abbreviations: LVX, levofloxacin; CIP, ciprofloxacin; FEP, cefepime; TET, tetracycline; MEM, meropenem.

resistant mutants sequenced, the only changes observed in the genes examined were in the sequence of *nfxB*, the negative regulator of the MexCD-OprJ efflux pump (Table 5). In addition to the mutants selected on agar plates, three colonies were isolated from the 24-h time point of the 4× MIC killing kinetics experiment. All three of these were found to also contain mutations in *nfxB* (data not shown). In the mutant *nfxB* genes, the genetic alterations, which included internal deletions and single-nucleotide changes that result in premature stop codons, are predicted to result in loss of function for this negative regulator of the MexCD-OprJ efflux system. Preliminary confirmation of *mexCD-oprJ* overexpression was obtained by quantitative RT-PCR (see Fig. S2 in the supplemental material). Three *nfxB* mutants were assessed, and based on the relative numbers of RNA amplification cycles, both the *mexC* and *oprJ* genes were found to be overexpressed roughly 15- to 20-fold over the levels in the parent strain PAO1.

As expected, when the same set of potential resistance genes were sequenced on mutants selected on levofloxacin, the results yielded "classic" first-step fluoroquinolone mutations in the quinolone resistance-determining regions (QRDR) of gyrase (Table 5). Sequencing of the gyrase, topoisomerase IV, and efflux pump regulator genes of the levofloxacin-resistant mutants identified T83I mutations in GyrA and E470D mutations in GyrB. Whereas the fluoroquinolone MIC values were increased by 8- to 16-fold, the NBTI 5463 MIC values actually decreased in these fluoroquinolone-resistant mutants.

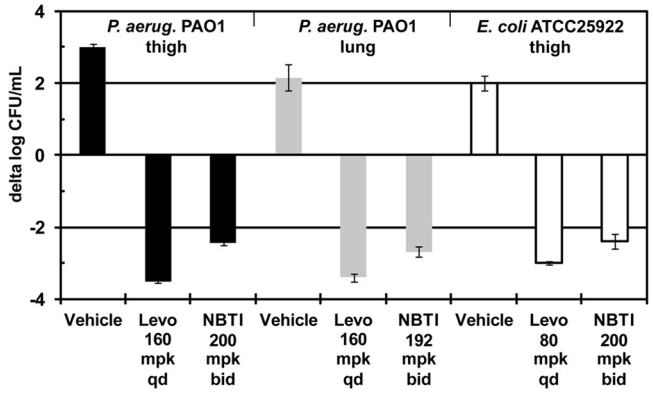

FIG 4 *In vivo* efficacy in thigh and lung models. The plots depict the change in CFU/ml, relative to the starting inoculum, after 24 h of infection with treatment with either vehicle (5% dextrose with lactic acid, pH 5.0), levofloxacin, or NBTI 5463. Error bars represent standard errors of the mean for each dose group of animals. mpk, mg/kg.

NBTI 5463 effect in mouse infection models. NBTI 5463 exhibited a dose-dependent response in both the neutropenic thigh and lung models of *P. aeruginosa* infection. At the start of therapy for the thigh and lung, mice were infected with 5.56 ± 0.06 and 6.15 ± 0.13 $\log_{10}$ CFU *P. aeruginosa* PAO1, respectively. In both models, mice receiving vehicle reached bacterial burdens of greater than 9.0 $\log_{10}$ CFU prior to 24 h and were sacrificed for ethical reasons. Figure 4 depicts the response to the top dose of NBTI 5463 compared to vehicle and levofloxacin. In both *P. aeruginosa* models, the top doses resulted in greater than a 2 $\log_{10}$ decrease in bacterial burden relative to that with the starting inoculum. As seen in Fig. 4, this was comparable to the decrease achieved in the *E. coli* ATCC 25922 neutropenic thigh model.

NBTI 5463 was also assessed in a neutropenic thigh model of *E. coli* infection using a clinical isolate, ARC1710. Table 6 shows the 24-h free AUC/MIC ratios necessary to achieve a static effect and reductions in CFU burdens of 1 $\log_{10}$ and 2 $\log_{10}$ for thigh and lung infections with *P. aeruginosa* PAO1 and for thigh infection with *E. coli* ARC1710.

NBTI 5463 was also evaluated in a mouse model of ascending urinary tract infection caused by *P. aeruginosa* and *E. coli*. NBTI 5463 showed a statistically significant response against *P. aeruginosa* VL098 in both kidney and bladder starting at a dose of 10 mg/kg/day (Table 7). Against *P. aeruginosa*, the maximum reductions in kidney and bladder bacterial burdens seen at 100 mg/kg/day were greater than 5.5 $\log_{10}$ and 1.1 $\log_{10}$ in kidney and bladder,

TABLE 6 *In vivo* efficacy in neutropenic mouse models of *P. aeruginosa* and *E. coli* following intraperitoneal dosing of NBTI 5463

PD magnitude	AUC/MIC ratio[a]		
	P. aeruginosa ARC545 (PAO1)		*E. coli* ARC1710, thigh
	Thigh	Lung	
Stasis	16	5	7
−1 log	24	9	11
−2 log	32	22	26

[a] Free-fraction corrected AUC/MIC required for the indicated magnitude of pharmacodynamic response relative to CFU/ml of the starting inoculum.

TABLE 7 *In vivo* efficacy in immunocompetent ascending urinary tract infection model of *P. aeruginosa* VL-098

Treatment (mg/kg/day)	Kidney		Bladder	
	n^a	Log_{10} CFU/g (mean ± SD)	n	Log_{10} CFU/g (mean ± SD)
Vehicle	7	5.51 ± 0.14	9	3.31 ± 0.11
Gentamicin (10)	10	5.24 ± 0.12[b]	10	2.84 ± 0.09[b]
NBTI 5463 (10)	9	4.16 ± 0.32[c]	9	2.51 ± 0.13[c]
NBTI 5463 (30)	2	2.24 ± 0.19[c]	9	2.38 ± 0.12[c]
NBTI 5463 (100)	0	Not detectable	9	2.02 ± 0.10[c]

[a] Number of animals out of 10 with detectable bacterial counts.
[b] Not statistically significant versus vehicle.
[c] Statistically significantly different from result for vehicle ($P < 0.05$).

respectively. Significant reductions were also demonstrated with *E. coli* VL-229 (data not shown).

DISCUSSION

One direction of the evolution of the NBTI series has been to improve activity against Gram-negative pathogens while minimizing the potential for inhibition of the hERG cardiac channel. In the present report, the microbiological, biochemical, and animal pharmacology properties of one of these compounds, NBTI 5463, is presented. Promising activities as measured by MIC values against multiple species of Gram-negative clinical isolates were observed. When tested against a small set of MDR isolates of *P. aeruginosa*, NBTI 5463 was found to exhibit good activity in the low, single-digit MIC range. The compound demonstrated concentration-dependent rapid bactericidal activity at the higher concentrations above the MIC against *P. aeruginosa* through the first 8 h in time-kill studies.

Earlier compounds in the series with Gram-positive activity demonstrated inhibition of *E. coli* topoisomerase II enzymes (16, 17). In the present case, again potent *in vitro* inhibition of both *E. coli* gyrase and topoisomerase IV was demonstrated with the NBTI compound. In contrast to fluoroquinolones, NBTI 5463 did not stabilize the formation of a double-strand DNA cleavable complex with DNA. Release of single-strand nicked DNA was observed, and creation of nicked DNA may be directly cytotoxic enough to promote cell killing. On the other hand, merely by trapping the enzyme bound to DNA, topoisomerase inhibitors can induce collisions between the topoisomerases and the rest of the DNA replication machinery (31, 32). Even though the bound DNA may not be cleaved with double-strand breaks, with the NBTI bound the topoisomerase is still locked onto the DNA, and a collision with the replication fork can start a chain of responses that leads to cell death (31). In this manner, even though NBTI 5463 possesses a mode of inhibition different from that of fluoroquinolones, the resultant antibacterial activity is largely similar. The lower rate of killing by NBTI 5463 may result from either the indirect effects that link a catalytic inhibitor to a cidal response or, alternatively, the weaker potency against gyrase than topoisomerase IV. Fluoroquinolone work has shown that inhibition of gyrase leads to a more rapid cidal response than topoisomerase IV inhibition (32). Other bacterial topoisomerase inhibitors that appear to act on the DNA-bound form of the enzymes have been reported, yet they do not promote double-strand DNA cleavage complex formation. Among the gyramides (33), ES-1273 (34), and 5,6-bridged dioxinoquinolones (35), the last series also displayed slower killing kinetics in *E. coli* than fluoroquinolones. However, potencies against the topoisomerase IV enzyme were not reported.

Resistant mutants were selected with NBTI 5463 at rates in the low 10^{-8} range in *P. aeruginosa*. From several strains, the target topoisomerase genes and several efflux pump regulator genes were sequenced. In all cases, likely loss-of-function mutations (point mutations, deletions, and stop codon insertions) were found in the *nfxB* gene, the negative regulator of the MexCD-OprJ efflux pump. Additional compounds in the NBTI series (not shown) were also found to exclusively select similar types of *nfxB* gene mutations, with a total of 14 different resistant strains selected, sequenced, and characterized from three NBTI compounds. NfxB is a helix-turn-helix DNA binding protein, and multiple changes in the sequence of the NfxB regulator protein have been associated with increased expression of the MexCD-OprJ efflux system (36, 37). Overexpression of MexCD-OprJ is associated with increases in fluoroquinolone and cefepime MIC values (37), both of which were noted with our NBTI 5463-selected resistant mutants. In contrast, *nfxB* mutations are known to increase susceptibility to compounds such as meropenem through decreased expression of the MexAB efflux pump outer membrane component OprM, as well as other cell envelope changes (37, 38). Since serious Gram-negative infections are often treated with multiple antibiotics, this finding suggests that a potential therapeutic strategy could be an NBTI compound in combination with a compound exhibiting increased susceptibility due to *nfxB* mutations.

Selection of resistance to levofloxacin yielded mutants with well-known resistance mutations in *gyrA* and *gyrB* (39). In this case, the increased MIC values were limited to fluoroquinolones. It was also noted that there was an increased susceptibility to the NBTI compound with these gyrase-based resistance mutations in *Pseudomonas*. This is another indication, in addition to the biochemical data, that NBTI compounds interact with the topoisomerase targets in a fashion distinct from that of fluoroquinolones. Further investigations to understand the nature of the increased susceptibility and the binding site of the NBTI compounds on gyrase are under way.

The *in vitro* and *in vivo* efficacies of non-NBTI antibiotics targeting the topoisomerase machinery have been correlated with the pharmacokinetic/pharmacodynamic index of AUC/MIC ratio (40–42). Previous work in the hollow-fiber system with an NBTI compound demonstrated that this index was also the driver for efficacy when tested against *S. aureus* (25). The free AUC/MIC ratios required for efficacy with NBTI 5463 against *E. coli* and *P. aeruginosa* in the neutropenic thigh model were lower than those demonstrated with fluoroquinolones (40, 41). NBTI 5463 also showed significant efficacy against *P. aeruginosa* in both the murine lung model and an ascending urinary tract infection model, indicating the ability of NBTI 5463 to penetrate these tissues.

NBTI 5463, described in this report, showed promising activity against *E. coli* and *P. aeruginosa*. Given the recent focus on the problems of infections by MDR Gram-negative bacteria, additional therapies are sought for these problematic pathogens (43). The NBTI compounds, as exemplified by NBTI 5463, offer a potentially novel compound series for further development to engage the bacterial topoisomerases, validated targets for antibiotic therapy.

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ERRATUM

NBTI 5463 Is a Novel Bacterial Type II Topoisomerase Inhibitor with Activity against Gram-Negative Bacteria and *In Vivo* Efficacy

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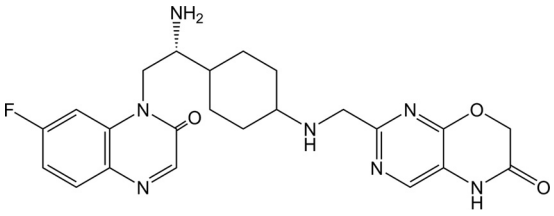
Volume 58, no. 5, p. 2657–2664, 2014. Page 2658: The structure of NBTI 5463 was incorrect as displayed in Figure 1 and should appear as shown below.



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