Novel Bacterial Topoisomerase Inhibitors with Potent Broad-Spectrum Activity against Drug-Resistant Bacteria

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ABSTRACT

The Novel Bacterial Topoisomerase Inhibitor class is an investigational type of antibacterial inhibitor of DNA gyrase and topoisomerase IV that do not have cross-resistance with the quinolones. Here, we report the evaluation of the *in vitro* properties of a new series of this type of small-molecule. Exemplar compounds selectively and potently inhibited the catalytic activities of *Escherichia coli* DNA gyrase and topoisomerase IV but did not block the DNA breakage-reunion step. Compounds showed broad-spectrum bactericidal activity against a wide range of Gram-positive and Gram-negative pathogens, including biodefence microorganisms, and *Mycobacterium tuberculosis*. No cross-resistance with quinolone-resistant *Staphylococcus aureus* and *E. coli* isolates was observed. Measured MIC_{90} values were 4 and 8 µg/mL against a panel of contemporary multidrug-resistant isolates of *Acinetobacter baumannii* and *E. coli*. In addition, representative compounds exhibited greater antibacterial potency than the quinolones against obligate anaerobic species. Spontaneous mutation rates were low, with frequencies-of-resistance typically $<10^{-08}$ against *E. coli* and *A. baumannii* at concentrations equivalent to four-fold the MIC. Compound-resistant *E. coli* mutants isolated following serial passage were characterised by whole-genome sequencing and carried a single Arg38Leu amino acid substitution in the GyrA subunit of DNA gyrase. Preliminary *in vitro* safety data indicate that the series shows a promising therapeutic index and potential for low hERG inhibition (IC_{50} >100 µM). In summary, the compounds' distinct mechanism-of-action relative to the fluoroquinolones, whole-cell potency, low potential for resistance development and favourable *in vitro* safety profile warrant their continued investigation as potential broad-spectrum antibacterial agents.

INTRODUCTION

Bacterial infections are becoming increasingly untreatable owing to the rapid emergence of multidrug resistance as well as the limited number of novel antibacterial agents in clinical development (1, 2, 3). The United States Centers for Disease Control and Prevention (CDC) recently identified 15 antibiotic-resistant microorganisms as posing a threat to human health classified as 'Urgent' or 'Serious' (4). Prominent amongst this set are antibiotic-resistant strains of the 'ESKAPE' group of species (1), such as carbapenem-resistant Enterobacteriaceae (CRE), multi-drug-resistant (MDR) *Acinetobacter*, MDR *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE). Also in the list are the Gram-positive anaerobe *Clostridium difficile*, drug-resistant *Neisseria gonorrhoeae* and drug-resistant tuberculosis.

The urgent need to discover and develop new antibacterial agents to counter the threat of drug-resistant infections is widely recognised. Research efforts over the past few years have focused on the development of novel classes of antibacterials with a dual-targeting mechanism-of-action distinct from currently-used antibiotics, with the twin objectives of avoiding cross-resistance and reducing the emergence of *de novo* resistance. The essential bacterial type II topoisomerase enzymes, DNA gyrase and topoisomerase IV, are well-validated drug targets for antibiotic pharmacology by the (fluoro)quinolone and aminocoumarin classes (5, 6, 7, 8). These enzymes are responsible for introducing negative supercoils into DNA and for the decatenation of DNA. The high degree of sequence similarity between DNA gyrase and topoisomerase IV offers the prospect of multi-targeting with a single pharmacophore (9, 10). Despite the now widespread resistance to the quinolones, the type II topoisomerases continue to provide opportunities for antibacterial discovery based on exploiting novel binding interactions between new chemical ligands and the target enzymes in order to bypass mutations associated with quinolone resistance. Selected examples of this strategy are the 2-aminoquinazolinedione (11), the isothiazoloquinolone (12), the spiropyrimidinetrione (13) and the novel tricyclic topoisomerase inhibitor (14) classes.

One emerging class of non-quinolone inhibitors of DNA gyrase and topoisomerase IV is the Novel Bacterial Topoisomerase Inhibitor (NBTI) type. NBTI molecules bind a site distinct from, but adjacent to, the catalytic centre of DNA gyrase/topoisomerase IV that is occupied by the quinolones (15). Consequently, NBTI compounds retain potency against quinolone-resistant isolates. Structurally, NBTI molecules comprise a northern head moiety that interacts with the DNA, a central linker portion, and a southern group that binds to the enzymes. A number of advanced NBTI molecules have been described in the literature, including NXL101 (16), AZD9742 (17), NBTI 5463 (18) and gepotidacin, (19), which is currently undergoing Phase II human clinical evaluation. The NBTI pharmacophore has been associated with cardiovascular and other safety liabilities (17, 20, 21, 22, 23). A key aim in the development of NBTIs, therefore, is achieving broad antibacterial potency, including against challenging Gram-negative pathogens, whilst maintaining satisfactory pharmacokinetics and safety margins.

Towards this goal, Redx Pharma recently disclosed a new series of NBTI type compounds characterised by a novel tricyclic LHS moiety (A. Ratcliffe, I. Cooper, M. Pichowicz, N. Stokes, C. Charrier, 18 February 2016, International Patent Application Number WO 2016/024096). The chemical structures of six selected compounds from this series are displayed in Figure 1. The purpose of this present study was to undertake a detailed *in vitro* biological evaluation of exemplar compounds from the series. Specifically, their ability to inhibit DNA gyrase and topoisomerase IV activities; their whole-cell potency against panels of wild-type and quinolone-resistant bacteria, including clinically-important anaerobes and biodefence organisms; and their *in vitro* safety profiles were assessed and are reported.

MATERIALS AND METHODS

Reagents and media. Proprietary compounds were prepared at Redx Pharma as described in International Patent Application WO 2016/024096. Reference antibiotics were purchased from Sigma Aldrich (Dorset, UK). Bacteriological media were purchased from Oxoid Ltd (Basingstoke, UK).

Bacterial strains. The bacteria used in this study were obtained from the American Type Culture Collection (ATCC, Middlesex, United Kingdom), the Network on Antibacterial Resistance in *Staphylococcus aureus* (Manassas, Virginia) or the Coli Genetic Stock Center (New Haven, Connecticut). *Escherichia coli* strains MG1655 WT, MG1655 S83L and MG1655 D87G were provided by Professor Tony Maxwell (John Innes Centre, Norwich, United Kingdom). *E. coli* ECCPX1-SP25 was selected and characterised at Redx Pharma by the serial passage of *E. coli* ATCC 25922 in the presence of ciprofloxacin as described (V. Savage, C. Charrier, N. Stokes, 18 February 2016, International Patent Application Number WO 2016/024098).

DNA supercoiling, decatenation and cleavage complex. DNA supercoiling, decatenation and cleavage complex assays were all performed by Inspiralis Ltd (Norwich, United Kingdom) using a gel-based assay format. Briefly, one unit of *E. coli* DNA gyrase was incubated with 0.5 µg of relaxed pBR322, and one unit of topoisomerase IV or human topoisomerase II was incubated with 200 ng kDNA, all in a reaction volume of 30 µL at 37 °C for 30 min in the presence of a series of concentrations of the test compound. Human topoisomerase II activity was assessed in the presence of 100 µM of test compound. Supercoiling reactions were conducted under the following conditions: 35 mM Tris.HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM Spermidine, 1 mM ATP, 6.5% (w/v) glycerol and 0.1 mg/ml BSA. *E. coli* topoisomerase IV decatenation reactions were conducted under the following conditions: 50 mM HEPES-KOH (pH 7.6), 100 mM potassium glutamate, 10 mM magnesium acetate, 10 mM dithiothreitol, 1 mM ATP and 50 µg/ml BSA. Inhibition of human topoisomerase II decatenation activity was assessed as described previously (Savage *et al*, 2016). Reactions were stopped

using 30 µL chloroform/iso-amyl alcohol (26:1) and 20 µL Stop Dye (40 % sucrose, 100 mM Tris.HCl [pH 7.5], 1 mM EDTA, 0.5 µg/mL bromophenol blue). Topoisomers were visualised by ethidium bromide staining, resolved and quantified by gel electrophoresis and the band intensities analysed by gel documentation equipment (Syngene, Cambridge, UK) and quantified using Syngene Gene Tools software. Raw data were converted to a percentage of the inhibitor-free control and were analysed using SigmaPlot Version 12.5. Non-linear regression was used to calculate the half-inhibitory concentrations (IC_{50}). The human topoisomerase II inhibitor, etoposide, was used as a positive control for inhibition for this assay. For cleavage complex assays, compounds were tested at 100 µM in a final DMSO concentration of 1% (v/v). *E. coli* DNA gyrase (one unit) was incubated with 0.5 µg of supercoiled pBR322 DNA at 37 °C for 30 min. Reactions were performed in a volume of 30 µL using the following conditions: 35 mM Tris.HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM Spermidine, 6.5% (w/v) glycerol and 0.1 mg/mL BSA. Following this, reactions were incubated for 30 min with 0.2 % SDS and 0.5 µg/µL proteinase K. Reactions were stopped in the same manner as for the supercoiling and decatenation assays. Topoisomers and cleavage products were visualised by gel electrophoresis. Cleavage products were expressed as a percentage of the fully supercoiled inhibitor-free control as described for the supercoiling and decatenation assays.

Antibacterial susceptibility testing. MICs were determined by the broth microdilution procedure according to the guidelines of the Clinical and Laboratory Standards Institute M07-A10 (24). The broth microdilution method involved a two-fold serial dilution of compounds in 96-well microtitre plates, giving a typical final concentration range of 0.25-128 µg/mL and a maximum final concentration of 1% DMSO. Strains were grown in cation-adjusted Müller-Hinton broth (CA-MHB) or agar (CA-MHA) with or without 5% lysed horse blood at 37°C in an ambient atmosphere, in haemophilus testing medium broth at 37°C in an ambient atmosphere, or in gonococcal broth or agar supplemented with Vitox at 37°C in an atmosphere containing 5% CO_2. The MIC was determined as the lowest concentration of compound that inhibits

visible growth following a 16-24 h incubation period. For *Mycobacterium tuberculosis*, a fluorescent reporter strain of H37Rv was used and the MIC was determined by measuring the optical density (OD_{590}) or fluorescence (Ex 560 nm/Em 590 nm) after five days of growth in 7H9 broth with 10% v/v OADC supplement and 0.05 % w/v Tween 80 in the presence of test compound with a final DMSO concentration of 2 %. MIC_{90} determination was performed at IHMA Europe Sàrl (Epalinges, Switzerland) with a selection of clinical isolates collected between 2012 and 2014. Bacteria were obtained from a variety of infection types and geographical locations including at least 25% highly drug-resistant isolates (resistant to at least seven out of amikacin, aztreonam, cefepime, ceftazidime, ceftriaxone, colistin, gentamicin, imipenem/meropenem, levofloxacin, piperacillin-tazobactam and tetracycline) and with a selection of 10 different species of anaerobes, including 113 isolates collected in 2015 from diverse geographical origins. MICs were performed using frozen 96-well antibacterial panels prepared by broth microdilution in line with the guidelines of the Clinical and Laboratory Standards Institute M11-A8 (25), giving a final compound concentration range of 0.004 to 64 µg/mL. The inoculum size was 5×10^5 CFU/mL and 5×10^7 CFU/mL for the aerobic and anaerobic strains, respectively. The testing plates for anaerobes were incubated for 48 h at 35 °C with 5% CO_2 in an anaerobic cabinet (Whitley A35 anaerobic workstation, Don Whitley Scientific). MICs were read visually and values were reported as MIC_{90} for inhibition of 90% of the isolates. MIC testing of *Bacillus anthracis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Francisella tularensis* and *Yersinia pestis* was undertaken by Southern Research (Birmingham, Alabama). Assay medium was CA-MHB (supplemented with 2% IsoVitalex in the case of *F. tularensis*). Cultures were incubated in the presence of compound for up to 24 h, up to 38 h (*F. tularensis*) or 24-48 h (*Y. pestis*).

Synergy/antagonism experiments. Antibacterial combinations were assessed using a two-dimensional checkerboard MIC method. Interpretation of the fractional inhibitory concentration index (FICI) was as described by Odds (26). Results shown are representative of at least two independent experiments.

Time-of-kill. The rate of bactericidal activity of compounds was determined against *A. baumannii* NCTC 13420 at 4 × MIC according to guidelines of the Clinical and Laboratory Standards Institute M26-A (27). *A. baumannii* was cultured overnight at 37°C, diluted in fresh CA-MHB and grown to exponential phase (OD_{600nm} = 0.3). Cultures were then adjusted to 0.5 McFarland units (1-2 × 10^8 CFU/mL) before addition of compound to give a final concentration of 4 × MIC. Samples were taken at 0, 0.5, 1, 3, 6 and 24 h, serial diluted and plated onto MHA, followed by overnight incubation at 37°C. The following day, colonies were enumerated to determine CFU/mL.

Frequency of resistance. Overnight cultures of bacteria were grown from single colonies in CA-MHB. The following day, samples of the neat cultures were spread onto CA-MHB containing compound at the concentrations indicated. To determine the number of viable cells in the inoculum, samples of the overnight cultures were serially diluted in phosphate-buffered saline (PBS) and plated on compound-free CA-MHA. Plates were incubated for up to 48 h and the colonies were enumerated. The spontaneous frequency of resistance (FoR) was calculated by dividing the number of resistant colonies (CFU/mL) by the total number of viable cells (CFU/mL).

Selection of resistant mutants by serial passage. Resistant mutants were selected by serial passage carried out using the broth microdilution method. Following MIC determination the culture representing 0.25 × MIC was used to inoculate the subsequent until the desired level of resistance was achieved. At this point, clones were isolated and the MIC confirmed as described above.

Whole-genome sequencing. Genomic DNA (gDNA) was extracted from the resistant strains using the PurElute Bacterial Genomic Kit (Edge BioSystems, Gaithersburg, Maryland). The gDNA was purified according to the manufacturer's instructions. Whole genome sequencing was performed at the Next Generation Sequencing facility at the University of Leeds (Leeds,

United Kingdom). Purified gDNA was used to create whole genome libraries using NEBNext Ultra kit and 150 bp paired end read sequence data were produced using an Illumina HiSeq 3000. Read data were stored as FASTQ files and then adaptor sequences were removed using cutadapt software (Version 1.8). Data for the wild-type strain was used to construct a reference genome sequence using the CLCBio genome assembler (Version 8.0.1). Sequence data for each sample, including the progenitor strain, were aligned to the published *E. coli* ATCC 25922 genome using BWA (Version 0.7.12); aligned data and were sorted using Samtools6 (Version 1.2). Variants were identified using VarScan (Version 2.3.7) using the *E. coli* ATCC 25922 assembled genome as the reference sequence. The resulting data provided coverage of >100 reads across the genome. Single nucleotide polymorphisms (SNPs), insertions and deletions were identified that were prevalent in ≥95% of the reads compared with the progenitor strain.

Cytotoxicity testing. HepG2 cells (ATCC HB-8065) were seeded at a density of 20,000 cells per well and incubated for 24 h at 37°C in an atmosphere of 5% CO_2. Cells were then exposed to a doubling dilution series of the test compound. After 24 h of incubation, the viability of the cells was determined using CellTiter-Glo® (Promega, WI, USA), according to the manufacturer's instructions. Each experiment was carried out in duplicate and the results reported as the average concentration of test compound inhibiting 50% of cell viability (IC_{50}).

hERG Inhibition. Inhibition of the human *Ether-a-go-go* Related Gene (hERG) cardiac potassium (K^+) ion channel was determined in a transfected Chinese Hamster Ovary K1 (CHO) cell line using IonWorks patch clamp electrophysiology (28).

LogD measurements. Partitioning of compounds between 1-octanol and 0.1 M phosphate buffer (pH 7.4) was measured using the shake-flask method (29).

RESULTS

Inhibition of target activity *in vitro*. The five compounds tested potently inhibited both *E. coli* DNA gyrase and topoisomerase IV enzymes, consistent with a dual-targeting mechanism-

of-action (Table 1). Ciprofloxacin was selected as a representative quinolone and tested in parallel for comparison. REDX05777, REDX06181, REDX06213, REDX07623 and REDX07638 produced a range of IC_{50} values comparable with ciprofloxacin in the supercoiling assay, while all five compounds showed approximately 10-fold lower IC_{50} values than ciprofloxacin in the decatenation assay. Stabilisation of the DNA gyrase cleavage complex was observed in the presence of ciprofloxacin (35% at 100 μM), while all Redx compounds showed little or no stabilisation of this complex at the same concentration. Taken together, these results indicate that the Redx compounds potentially have a more balanced dual targeting activity than ciprofloxacin and a distinct mechanism-of-action. Similar to ciprofloxacin, Redx compounds showed a high degree of selectivity for the bacterial enzymes over the homologous mammalian enzyme, human topoisomerase II, with approximately two orders of magnitude difference in the measured IC_{50} values (Table 1).

Bacterial susceptibility profile. Bacterial susceptibility profiling of the NBTI compounds indicates that the series has broad-spectrum activity against clinically-important Gram-negative and Gram-positive pathogens, including those of the 'ESKAPE' group of microorganisms (Table 2). Compounds from the series were generally more potent against the Gram-positive species. Fastidious Gram-negatives, such as *Haemophilus infuenzae* and *Neisseria gonorrhoeae*, as well as non-fastidious Gram-negatives species were susceptible to the compounds. Of the non-fastidious Gram-negatives, *Acinetobacter baumannii* and *E. coli* were generally more susceptible than *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Representative compound REDX05777 also potently inhibited the whole-cell proliferation of *Mycobacterium tuberculosis* (Table 2). The exemplar compound REDX07638 was tested against a set of five biothreat micro-organisms that included the aetiological agents of anthrax, Glanders, meliodosis, tularaemia and the plague (Table 3). REDX07638 inhibited all five Gram-positive and Gram-negative species, with *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis* being the most susceptible.

Compounds retained potency against the *E. coli* strains, MG1655 S83L and MG1655 D87G, carrying the Ser83Leu and Asp87Gly mutations in the GyrA subunit of DNA gyrase that are associated with quinolone resistance. In all cases the MIC for the mutants was within one-doubling-dilution either side of the MIC for the isogenic parent strain *E. coli* MG1655. By contrast, the MIC of ciprofloxacin increased 8-16-fold against the MG1655 S83L and MG1655 D87G strains compared to the isogenic parent (Table 2). *E. coli* ECCPX1-SP25 is a ciprofloxacin-resistant mutant derived from the wild-type parent strain ATCC 25922. The MIC of ciprofloxacin against this strain is elevated 1024-fold relative to the parent strain. In comparison, the activity of Redx compounds against *E. coli* ECCPX1-SP25 was within 2-to-8-fold of strain ATCC 25922 (Table 2). Taken together, these results indicate a lack of cross-resistance of the NBTI series with the quinolone class of antibiotics.

A selection of Redx compounds were tested against a panel of recent multidrug-resistant (MDR) and quinolone-resistant Gram-negative clinical isolates (Table 4). All three compounds tested showed antibacterial activity against *E. coli* with a MIC_{90} of 4 or 8 µg/mL. Similarly, good activity was observed with REDX07623, REDX06213 and REDX06276 against *A. baumannii* with MIC_{90} of 4 or 8 µg/mL. The MIC_{90} values observed for these NBTI compounds against *E. coli* and *A. baumannii* were lower than those obtained for the fluoroquinolone antibiotic levofloxacin (16 µg/mL). REDX06276 was the most active compound from this series against the *K. pneumoniae* panel with a MIC_{90} of 16 µg/mL, comparable to levofloxacin. Activity against *P. aeruginosa* and *E. cloacae* was observed at 32-to-64 µg/mL for all compounds tested, which was similar to the MIC_{90} values obtained for levofloxacin.

Finally, compounds were tested for activity against species of clinically-significant obligate anaerobic Gram-positive and Gram-negative bacteria. Antibiotics of the quinolone class have generally shown poor-to-moderate *in vitro* antibacterial potency against anaerobic bacteria relative to other classes of antibiotics and compared with their potency against aerobic bacteria (30, 31). Metronidazole and vancomycin were equally effective against a panel of recently-isolated anaerobes including the Gram-positive strains of *P. harei, P. anaerobius* and *C. perfringens* with MIC_{90} values lower or equal to 2 µg/mL (Table 5). Metronidazole, however,

was not active against *P. acnes* while the Redx compounds maintained activity with MIC_{90} values of 0.5 and 4 µg/mL. Similar to ciprofloxacin, the tested compounds showed reduced activity against the Gram-positive strains of *F. magna* and *P. micra* with MIC_{90} values 16-64 µg/mL. Against the Gram-negative isolates, Redx compounds showed activity at 2-to-16 µg/mL against the four bacterial species tested. Although metronidazole displayed lower MIC_{90} values (0.25-to-2 µg/mL), the NBTI compounds showed improved activity compared to ciprofloxacin and vancomycin.

Antibiotic combination studies. Since co-administration of antibiotics acting on different targets can enhance antibacterial activity, REDX07638 was tested in combination with ampicillin and delafloxacin against several Gram-negative strains including those conferring resistance to β-lactam and fluoroquinolone antibiotics. An FICI was calculated and interpreted as described previously (26). A FICI of 0.5 was calculated for REDX07638 in combination with ampicillin against β-lactam resistant *E. coli* NCTC 13476 indicating synergism. This combination showed an FICI of 2 against other β-lactam strains indicating no interaction. Additionally, REDX07638 in combination with delafloxacin showed an FICI of 0.75-1.25 against fluoroquinolone resistant (FQR) strains *E. coli* ECDEL-SP45 and *K. pneumoniae* ATCC 13439, indicating no interaction. These results suggest REDX07638 in combination with ampicillin or delafloxacin could be used given the lack of antagonism observed.

Time of kill. The rate of bactericidal activity was determined for REDX06213, REDX06276, REDX07623 and REDX07638 against *A. baumannii* NCTC 13420 at 4 × MIC. REDX06213 and REDX06276 both demonstrated bactericidal activity, showing a 3-log drop in CFU/mL at 1.56 and 1.06 h, respectively. A 3-log reduction in CFUs was not achieved within 24 h with REDX07623 and REDX07638 at 4 × MIC against this strain. All compounds showed regrowth at 24 h. Bactericidal activity of ciprofloxacin against *A. baumannii* has been documented in the literature and demonstrates a 3-log drop in CFU/mL at 4× MIC, with no significant regrowth at 24 h (32).

Selection of resistant mutants. In order to assess the propensity for the development of *de novo* resistance to this class of NBTI compounds, the spontaneous frequency of resistance to REDX06213, REDX06276, REDX07623 and REDX07638 was determined with *E. coli* strain ATCC 25922. No mutants could be isolated at concentrations equivalent to 4× MIC, yielding frequencies-of-resistance ranging from $<2.5 \times 10^{-9}$ to $<3.3 \times 10^{-9}$. By comparison, the frequency-of-resistance to ciprofloxacin at 4 × MIC was 7.8×10^{-8} for *E. coli* ATCC 25922. To confirm that the observed mutation frequencies were not species-specific, frequencies-of-resistance values were also determined for REDX06213, REDX07623 and REDX07638 in *A. baumannii* strain NCTC 13420. Again, no mutants were isolated, yielding frequencies-of-resistance between $<6.7 \times 10^{-8}$ and $<7.4 \times 10^{-8}$.

Next, *E. coli* ATCC 25922 was used in serial passage experiments with REDX06276 as a representative compound from this NBTI series. Ciprofloxacin and delafloxacin were used as comparator antibiotics. The MIC of ciprofloxacin increased up to 64 µg/mL after 25 passages with resistance observed at passage 23 (MIC ≥ 4 µg/mL). The MIC of delafloxacin, however, remained within 2-fold of the original MIC (0.5 µg/mL) up to passage 24, after which it increased steadily to reach 16 µg/mL (32-fold increase) at passage 45. The MIC of REDX06276 followed a comparable trend to delafloxacin with an increase up to 32-fold (MIC 64 µg/mL) at passage 45, at which stage the experiment was ended (Figure 2). Whole genome sequencing of the ciprofloxacin resistant mutant from passage 25 (ECCPX1-SP25) revealed Ser83Leu and Asp87Gly mutations in the GyrA subunit and a Glu84Lys mutation in the ParC subunit. The delafloxacin-resistant mutant at passage 45 had target gene mutations corresponding to Ala119Glu and Ala179Val amino acid substitutions in the GyrA subunit. The REDX06276-resistant mutant from passage 45 carried a single Arg38Leu substitution in the GyrA subunit.

***In vitro* safety profile.** Mammalian cytotoxicity testing with the HepG2 cell line revealed IC_{50} values that were higher than the corresponding MIC values observed with the ESKAPE

pathogens (Table 6). For some compounds the therapeutic window exceeded two orders of magnitude. *In vitro* testing showed the series to have a range of hERG activity (Table 6), with REDX07623 having an IC_{50} of 8.2 μM, whilst REDX6181 demonstrated reduced hERG inhibition with an IC_{50} of >100 μM. A correlation between logD and hERG activity was found with this series. Compounds with a lower logD appeared to have reduced hERG inhibition (Table 6).

DISCUSSION

In recent years the growing threat of drug-resistant bacterial infections and the lack of new antibiotics with a novel mechanism-of-action has caused global concern. Resistance of Gram-negative species to first line and last resort antibiotics has been reported worldwide and can

lead to untreatable infections and increased mortality (33). To address this unmet medical need, this study describes the *in vitro* assessment of an NBTI series with dual-targeting activity against bacterial DNA gyrase and topoisomerase IV and a different mechanism-of-action to clinically used fluoroquinolones. Redx compounds demonstrated potent, balanced inhibitory activity versus the two topoisomerase enzymes, with IC_{50} values ranging from 0.21 to 1.66 µM with *E. coli* DNA gyrase and between 0.10 and 1.17 µM against *E. coli* topoisomerase IV (Table 1). Inhibitory activity was more balanced than ciprofloxacin, which had IC_{50} values of 0.77 and 10.20 µM against *E. coli* gyrase and topoisomerase IV, respectively. This is in agreement with data recorded in the literature, which shows ciprofloxacin to have a preference for DNA gyrase in *E. coli* and topoisomerase IV in *S. aureus* (34). Importantly, selectivity over human topoisomerase II was found with bacterial enzymes showing an approximate 100-fold increase in sensitivity to Redx compounds. The formation of DNA cleaved complexes was limited with Redx compounds in comparison to the level observed with ciprofloxacin (Table 1). This indicates that the NBTI series described here has a different mechanism-of-action to ciprofloxacin, which stabilises double-stranded broken DNA strands, blocking re-ligation, the consequences of which are poisonous to the bacterial cell. Instead, the NBTIs described here interact with the topoisomerase and DNA prior to double strand breakage, which has been reported for other NBTI series (18).

Broad-spectrum antibacterial activity was found with this series against a panel of ESKAPE pathogens, the fastidious Gram-negative organisms *H. influenzae* and *N. gonorrhoeae*, as well as *M*. *tuberculosis* and important Gram-positive and Gram-negative biothreat pathogens (Table 2 and 3). Potency was maintained against FQR *E. coli* isolates with a single amino acid substitution in the GyrA subunit (Ser83Leu or Asp87Gly) with MICs within two-fold of the MIC for the isogenic parent strain. Additionally, compounds retained potency against the serial passage FQR mutant *E. coli* ECCPX1-SP25, with MICs increasing eight-fold or less, in comparison to ciprofloxacin which showed a 1024-fold increase in MIC. The retained potency of this series against FQR mutants supports the different mechanism-of-action to the fluoroquinolones indicated by the cleavage complex enzyme assay. Good antibacterial activity

was retained against a larger panel of MDR strains (25% levofloxacin resistant) of *A. baumannii* and *E. coli*, with MIC_{90} values of 4-8 μg/mL. A loss of potency was found against the other Gram-negative strains, *K. pneumoniae, E. cloacae* and *P. aeruginosa*, with MIC_{90} values of 16-32 μg/mL, although these were still equal to or superior to levofloxacin and other fluoroquinolones reported in the literature (35). In addition to good activity against ESKAPE pathogens, compounds demonstrated antibacterial activity against a panel of anaerobic pathogens including *Clostridium* and *Bacteroides* species (Table 2 and 5). In Europe, *C. difficile* is estimated to cause 250,000 infections and 14,000 deaths per annum, showing resistance to a large number of antibiotics including the fluoroquinolones (36). *Bacteroides* species are part of the mammalian gut microflora and can be opportunistic pathogens as well as a reservoir for resistance. They are also frequently resistant to a wide range of antibiotics, necessitating development of novel compounds that are effective against these species..

A representative compound, REDX07638, was tested in combination with ampicillin and delafloxacin against several resistant Gram-negative strains and showed no antagonism, suggesting that this series of compounds could potentially be used in combination therapy. This finding has been reported previously with other bacterial topoisomerase II inhibitors in combination with other antibiotics (37, 38, 39). Interestingly, REDX07638 and ampicillin demonstrated a synergistic interaction against *E. coli* NCTC 13476, but this was not consistently observed for other β-lactam resistant strains.

Rapid, bactericidal activity of the series was confirmed with REDX06213 and REDX06276 against *A. baumannii* NCTC 13420 at 4 × MIC, with both compounds causing a 3-log drop in CFU/mL at 1.56 and 1.06 h, respectively. The rate of bactericidal activity was similar or superior to that for ciprofloxacin and other NBTIs reported in the literature (32, 40, 41).

No mutants were raised against compounds tested at 4 × MIC with *A. baumannii* NCTC 13420 and *E. coli* ATCC 25922. By contrast, a mutation rate of 4.76×10^{-8} was obtained with *E. coli* ATCC 25922 against ciprofloxacin at equivalent multiples of its MIC. These results indicate a low potential for resistance development to this series and support the balanced dual-targeting

activity revealed by the supercoiling and decatenation assay data. Development of resistance to other NBTI series at 4 × MIC has been reported in the literature. Resistance rates of 5×10^{-8} were found with NBTI 5463 against *P. aeruginosa* PAO1, although sequencing showed no target gene mutations (18). Second- and third-step mutants had Asp82Glu and Asp82Glu plus Asp87Tyr point mutations in GyrA, respectively (42). These mutants showed no cross-resistance to the fluoroquinolones consistent with a differential binding mechanism between the NBTI and fluoroquinolone classes of topoisomerase inhibitors. Although no mutants were raised during the spontaneous frequency-of-resistance experiments against the compounds described here, whole genome sequencing of the *E. coli* REDX06276 serial passage mutant revealed a single Arg38Leu substitution in the GyrA subunit. This mutation has been reported previously and conferred resistance to 5,6-bridged quinolones, but not other quinolones (43). Similarly, no cross-resistance to ciprofloxacin was found with the *E. coli* REDX06276 serial passage mutant (data not shown).

Compounds in this series show a promising safety profile with HepG2 cytotox IC_{50} values of ≥32 μg/mL. Reduced hERG activity with the series has also been demonstrated, with IC_{50} values of >100 μM. During the optimisation of this series, efforts have been made to reduce hERG effects while retaining antibacterial potency. The addition of a fluorine atom in the southern group of REDX07623 appears to increase the hERG activity in comparison to its matched pair, REX06276, with IC_{50} values of 8.2 and >33 μM, respectively. Introduction of more polar groups to reduce the logD of a compound, thereby reducing hERG activity, has been reported for NBTIs previously in the literature (22). REDX06181, with the lowest logD of the compounds tested, showed the most attenuated hERG activity with an IC_{50} of >100 μM, although relative to other compounds with a higher logD, such as REDX07623, its antibacterial potency was reduced. A negative correlation between whole-cell antibacterial potency and hERG activity has been reported for other NBTI type compounds (22).

In summary, the NBTI series described here shows potent, balanced, dual-targeting inhibition of DNA gyrase and topoisomerase IV, with selectivity over human topoisomerase II. Data from

DNA cleaved complex experiments indicates the series has a different mechanism-of-action to the fluoroquinolones. The low mutation rate of Gram-negative strains to the compounds combined with the balanced inhibitory enzyme activity suggests resistance could be slow to develop during therapeutic use. Antibacterial activity was demonstrated against a wide panel of susceptible and drug-resistant bacterial species including the ESKAPE set of organisms, medically-important anaerobic species and other pathogens, including larger sets of MDR isolates thereof. Rapid, bactericidal activity was also demonstrated. These properties, in combination with the promising *in vitro* safety profile, warrants the further development of this NBTI series.

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TABLE 1 Inhibition of the DNA supercoiling and cleaved complex formation activities of *E. coli* DNA gyrase, the decatenation activity of *E. coli* topoisomerase IV, and the decatenation activity of human topoisomerase II by ciprofloxacin, REDX06181 and REDX06213.

	Ciprofloxacin	REDX 05777	REDX 06181	REDX 06213	REDX 07623	REDX 07638
E. coli DNA gyrase IC_{50} (μM)	0.77	0.29	1.47	1.66	0.21	0.23
E. coli DNA gyrase cleavage complex (% cleaved)	35.0	0	2.6	3.5	0	0
E. coli topoisomerase IV IC_{50} (μM)	10.20	0.25	1.17	0.14	0.10	0.10
Human topoisomerase II IC_{50} (μM)	500	>100	84	100	>100	100

TABLE 2 Bacterial susceptibility profile of NBTI compounds against reference bacterial strains and fluoroquinolone mutants.

Species and strain	MIC (µg/mL)						
	Ciprofloxacin	REDX 05777	REDX 06181	REDX 06213	REDX 06276	REDX 07623	REDX 07638
Acinetobacter baumannii NCTC 13420	64	2	16	0.12	0.25	0.25	0.25
Clostridium difficile ATCC 700557	16	n.d.	n.d.	2	n.d.	2	n.d.

Enterobacter cloacae NCTC 13406	0.015	8	8	2	1	2	2
Enterococcus faecalis ATCC 29212	1	2	2	1	0.5	1	1
Escherichia coli ATCC 25922	0.03	0.5	1	0.12	0.25	0.5	0.5
E. coli MG1655 WT	0.008	0.5	1	0.25	0.5	0.5	0.5
E. coli MG1655 S83L	0.12	0.5	0.5	0.25	0.25	0.25	0.25
E. coli MG1655 D87G	0.06	1	0.5	0.5	1	1	1
E. coli ECCPX1-SP25	32	4	1	1	0.5	1	2
Haemophilus influenzae ATCC 49247	0.008	4	4	2	2	4	2
Klebsiella pneumoniae ATCC 700603	0.25	16	32	8	8	8	8
Mycobacterium tuberculosis H37Rv	2.2	1.3	n.d.	n.d.	n.d.	n.d.	n.d.

Nesisseria gonorrhoeae ATCC 49226	0.004	4	16	2	1	2	2
Pseudomonas aeruginosa ATCC 27853	1	8	8	4	4	8	4
Staphylococcus aureus ATCC 29213	0.25	1	4	0.12	0.25	0.5	0.12
Streptococcus pneumoniae ATCC 49619	0.5	2	4	0.5	0.25	0.5	0.25

n.d., not determined

TABLE 3 Susceptibility profile of a panel of biodefence pathogens to REDX07638 and the comparator antibiotic doxycycline.

Species and strain	MIC (µg/mL)	
	Doxycycline	REDX07638
Bacillus anthracis Ames	0.03	0.5
Burkholderdia mallei China 7	0.12	8
Burkholderdia pseudomallei DD503	2	32
Burkholderdia pseudomallei K96243	2	32
Burkholderdia pseudomallei 1026b	1	16
Francisella tularensis SCHU S4	0.5	4
Yersinia pestis CO92	2	1

TABLE 4 MIC_{90} (µg/mL) of NBTI compounds and levofloxacin for a panel of recently isolated levofloxacin-resistant and multidrug-resistant clinical isolates.

Species (number of isolates)	MIC_{90} (µg/mL)			
	Levofloxacin	REDX06213	REDX06276	REDX07623
K. pneumoniae (42)	16	32	16	32
A. baumannii (43)	16	8	8	4
P. aeruginosa (42)	64	32	32	32

E. cloacae (41)	32	32	32	32
E. coli (43)	16	4	4	8

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772 **TABLE 5** Bacterial susceptibility profile of NBTI compounds and comparator antibiotics

773 against a panel of recent clinical isolates of 10 anaerobic bacterial species.

Species (number of isolates)	MIC_{90} (µg/mL)				
	Metronidazole	Vancomycin	Ciprofloxacin	REDX06213	REDX07623
Clostridium perfringens (11)	1	0.5	0.5	2	2
Finegoldia magna (12)	0.5	0.5	32	16	32
Parvimonas micra (12)	1	1	16	64	64

Peptostreptococcus anaerobius (10)	0.5	0.5	16	0.25	1
Propionibacterium acnes (12)	>32	0.5	1	0.5	4
Peptoniphilus harei (12)	1	0.12	4	≤0.12	0.5
Bacteroides fragilis (12)	0.5	32	16	4	4
Bacteroides thetaiotaomicron (11)	1	64	64	16	8
Prevotella bivia (10)	2	>64	32	8	8
Prevotella melaninogenica (11)	0.25	>64	4	2	4

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778 **TABLE 6** *In vitro* safety profile of NBTI compounds and ciprofloxacin.

	REDX 05777	REDX 06181	REDX 06213	REDX 06276	REDX 07623	REDX 07638
HepG2 IC_{50} (µg/mL)	>64	>64	29.6	>64	>64	38
hERG IC_{50} (µM)	>33	>100	>33	>33	8.2	8.9
logD	0.65	-0.64	0.94	0.71	1.26	1.39

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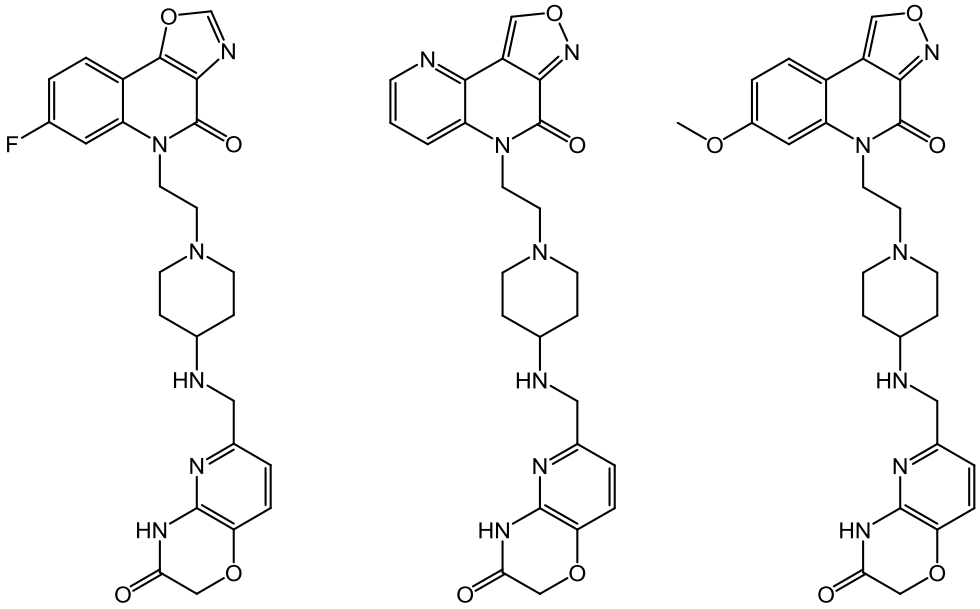
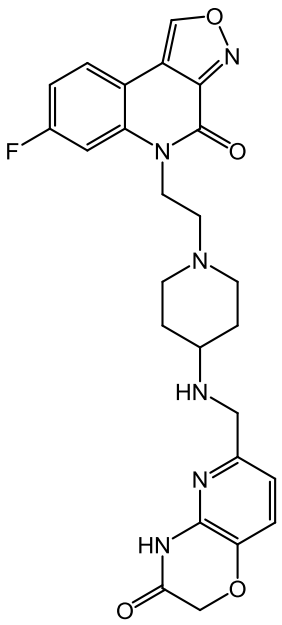
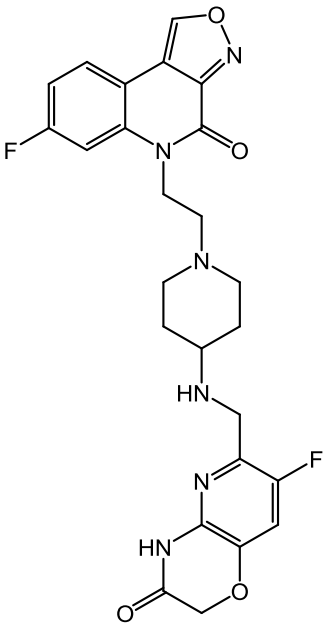
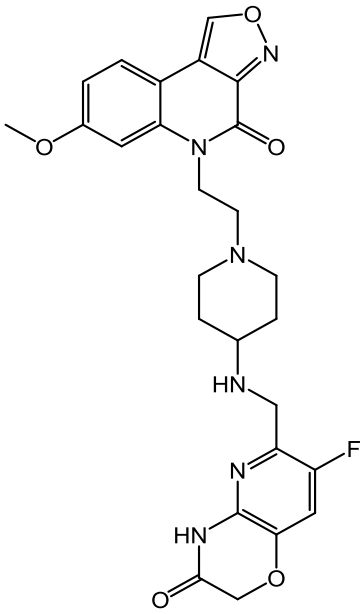
FIGURE 1 Chemical structures of the compounds described in this study.



REDX05777 REDX06181 REDX06213

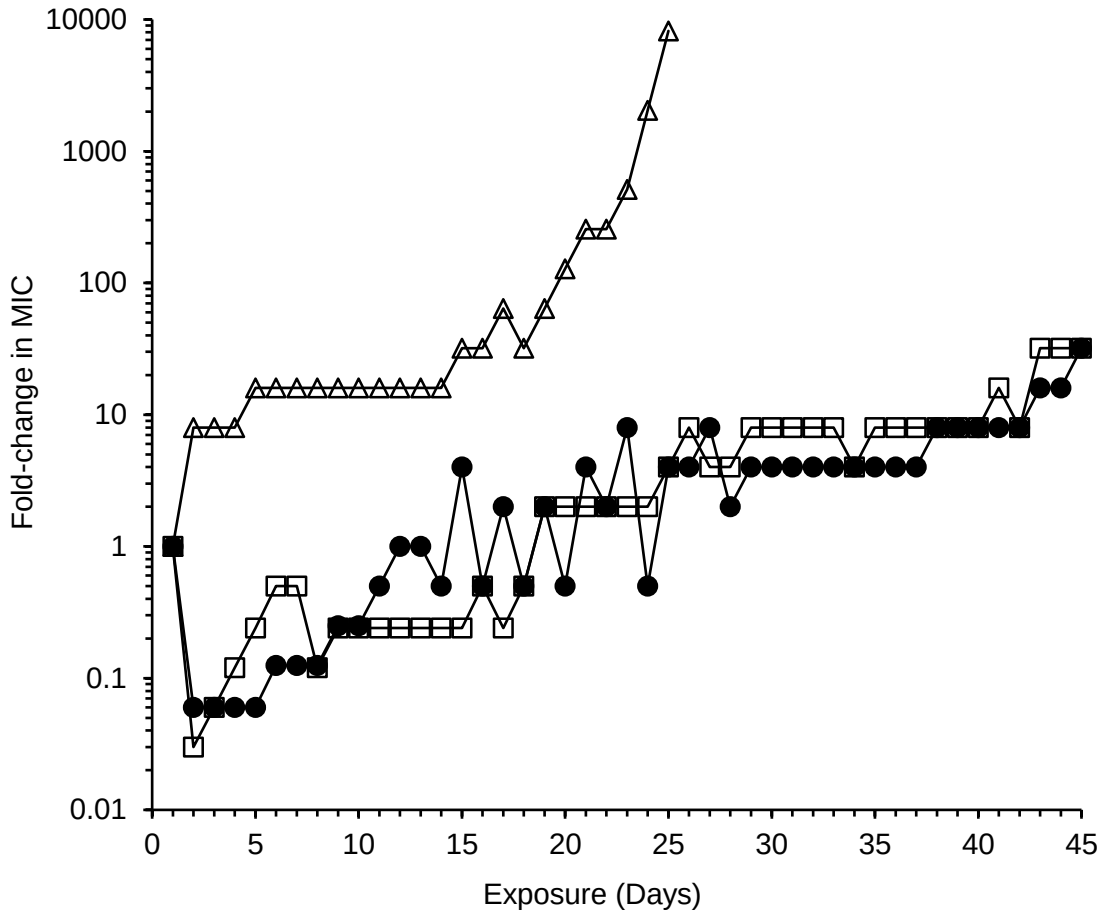






REDX06276 REDX07623 REDX07638

FIGURE 2 Isolation of drug-resistant mutants of *E. coli* ATCC 25922 by serial passage. Closed circles, REDX06276; open triangles, ciprofloxacin; open squares, delafloxacin.



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