***In Vitro* Biological Evaluation of Novel Broad-Spectrum Isothiazolone Inhibitors of Bacterial Type II Topoisomerases**

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**SYNOPSIS**

**Objectives:** Evaluate the *in vitro* biological properties of a novel class of isothiazolone inhibitors of the bacterial type II topoisomerases.

**Methods:** Inhibition of DNA gyrase and topoisomerase IV activity was assessed using DNA supercoiling and decatenation assays. MIC and MBC were determined according to CLSI guidelines. Antibacterial combinations were assessed using a two-dimensional checkerboard MIC method. Spontaneous frequency of resistance was measured at various multiples of the MIC. Resistant mutants were generated by serial passage at sub-inhibitory concentrations of antibacterials and genetic mutations were determined through whole genome sequencing. Mammalian cytotoxicity was evaluated using the HepG2 cell line.

**Results:** Representativeisothiazolone compound REDX04957 and its enantiomers (REDX05967 and REDX05990) showed broad-spectrum bactericidal activity against the ESKAPE organisms, with the exception of *Enterococcus* spp., as well as against a variety of other human bacterial pathogens. Compounds retained activity against quinolone-resistant strains harbouring GyrA S83L and D87G mutations (MIC ≤ 4 mg/L). Compounds inhibited the supercoiling activity of wild-type DNA gyrase and the decatenation function of topoisomerase IV. Frequency of resistance of REDX04957 at 4 × MIC was <9.1 × 10-09. Against a panel of recent multidrug-resistant (MDR) isolates, REDX05967 demonstrated activity against *A. baumannii* with a MIC50 and a MIC90 of 16 and 64 mg/L, respectively. Compounds showed a lack of cytotoxicity against HepG2 cells at 128 mg/L.

**Conclusions:** Isothiazolone compounds show potent activity against Gram-positive and Gram-negative pathogens with a dual targeting mechanism-of-action and a low potential for resistance development, meriting their continued investigation as broad-spectrum antibacterial agents.

**Introduction**

The continuous emergence of antibiotic resistance in both the clinical and community settings is a serious concern and an ever-increasing public health threat. A recent review on antibiotic resistance estimated that failing to tackle drug resistance will cause 10 million extra deaths a year across the world by 2050 and will cost the global economy up to $100 trillion.[1](#_ENREF_1) Of particular concern is the so-called ‘ESKAPE’ group of Gram-positive and Gram-negative bacterial pathogens, comprising Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter species.[2](#_ENREF_2) The lack of novel antibiotic classes since the beginning of the new millennium, the so-called ‘antibiotic innovation gap’, is a contributing factor to the repeated calls from various organisations for the development of new antibiotics.[3-5](#_ENREF_3) The scarcity of new classes of drugs, particularly for Gram-negative bacteria, highlights the urgent need to promote and stimulate antibiotic research and development.[6](#_ENREF_6), [7](#_ENREF_7)

There are numerous reasons for the discovery void and the lack of novel antibiotic classes. These include the lack of credible, new molecular targets once expected with the advent of the genomic era and the limitations of conventional approaches such as target-based or phenotypic high-throughput screening.[8](#_ENREF_8), [9](#_ENREF_9) Indeed, engagement of a specific molecular target does not necessarily translate to growth inhibition and/or killing of a bacterial cell, while the high genetic diversity and potential different physiological states amongst the microbial pathogens responsible for an infection requires a complex combination of physicochemical and pharmaceutical properties for a novel antibiotic. Because of these technical challenges, combined with high attrition rates, long development timelines and costly clinical trials, incremental modification of existing antibiotics has become an attractive strategy. However, this may not provide a long-term solution to the underlying mechanisms of resistance.[10](#_ENREF_10), [11](#_ENREF_11)

The urgent need for novel broad-spectrum antibiotics able to overcome existing resistance mechanisms in a range of bacterial species has been acknowledged by a variety of stakeholders and novel strategies to address the various challenges involved are currently being formulated.[1](#_ENREF_1), [7](#_ENREF_7), [9](#_ENREF_9) However, the implementation of such strategies will take time and novel therapeutic options are required urgently. Narrow spectrum antimicrobial agents and drug combination therapies that target the specific bacterial pathogens such as those listed by the US CDC as ‘urgent threats’ e.g. carbapenem-resistant Enterobacteriaceae, or ‘serious threats’ e.g. multidrug-resistant *Acinetobacter* and extended spectrum β-lactamase producing Enterobacteriaceae, provide alternative strategies in the fight against established and emerging antimicrobial resistance.[12](#_ENREF_12), [13](#_ENREF_13)

One strategy to discover new antibiotics is the design and optimisation of novel chemical inhibitors of validated targets. The bacterial type II topoisomerases, DNA gyrase and topoisomerase IV, are well-established targets for antibacterial chemotherapy in Gram-positive and Gram-negative pathogens.[14-16](#_ENREF_14) DNA gyrase is comprised of two protein subunits termed GyrA and GyrB. Topoisomerase IV comprises two subunits called ParC (GrlA) and ParE (GrlB). The quinolone class of antibiotics, such as ciprofloxacin, levofloxacin and moxifloxacin, targets the GyrA and ParC subunits of DNA gyrase and topoisomerase IV,[17](#_ENREF_17) respectively whilst the coumarins, exemplified by novobiocin, inhibit GyrB.[18-20](#_ENREF_18) Bacterial resistance to the quinolones is widespread. Quinolone resistance can result from target-based mutations in one of the four topoisomerase genes, the expression from extrachromosomal elements of proteins such as the Qnr proteins that disrupt quinolone-enzyme binding, or drug efflux via membrane-localised pumps.[21](#_ENREF_21) Target-mediated quinolone resistance is most frequently associated with mutations in the Ser83 and Asp87 residues in GyrA.[21](#_ENREF_21) These residues are important in forming hydrogen bonds with the water molecules of the water-Mg2+ ion bridge that is chelated by the keto acid groups of the quinolone.

Owing to the success of the topoisomerases as targets for broad-spectrum antibacterial drug discovery there have been various efforts to discover non-quinolone inhibitors of these enzymes that bypass target-mediated quinolone resistance by avoiding the formation of the water-metal ion interaction. Examples include the 3-aminoquinazolinediones[22](#_ENREF_22), [23](#_ENREF_23) and the novel bacterial topoisomerase inhibitor (NBTI) classes.[24](#_ENREF_24) Redx Pharma recently disclosed a series of chemically-novel isothiazolone-based small-molecule inhibitors of DNA gyrase and topoisomerase IV.[25](#_ENREF_25) Isothiazolone compounds have previously been associated with non-specific biocidal activity against bacteria, algae and fungi, however, their use has been limited to industrial applications for control of microbial growth and biofouling.[26](#_ENREF_26), [27](#_ENREF_27) This study describes inhibition of bacterial DNA gyrase and topoisomerase IV enzymes by a novel class of isothiazolone-based compounds and their potential clinical application. The chemical structure of the exemplar compound of this series, REDX04957 (*rac*-6-[4-[3-(dimethylamino)pyrrolidin-1-yl] phenyl]-7-ethyl-5H-isothiazolo[4,5-c]pyridine-3,4-dione) is shown in Figure 1. Here, the data from *in vitro* studies to evaluate the biological properties of REDX04957 and its two enantiomers are presented.

**Materials and Methods**

**Antibacterials**

Compounds REDX04957 and its enantiomers were synthesized and purified at Redx Pharma as described in International Patent Application WO2015/114317.[25](#_ENREF_25) Reference antibiotics were purchased from Sigma.

**Bacterial strains**

Bacteria used in this study were obtained from the ATCC, the Network on Antibacterial Resistance in *Staphylococcus aureus* (Manassas, Virginia) or the Coli Genetic Stock Center (New Haven, Connecticut). *Escherichia coli* MG1655 WT, *E*. *coli* MG1655 S83L and *E*. *coli* MG1655 D87G were kindly provided by Professor Tony Maxwell (John Innes Centre, Norwich, United Kingdom). The WHO L reference strain was generously provided by Professor Magnus Unemo (Örebro University). Strains *E*. *coli* ECCPX1-SP22 and *E*. *coli* ECCPX1-SP25 were created and characterised at Redx Pharma by serial passage of *E*. *coli* ATCC 25922 in the presence of ciprofloxacin as described in International Patent Application WO2016/024098.[28](#_ENREF_28)

**DNA supercoiling and decatenation**

Inhibition of the supercoiling function of purified DNA gyrase and the decatenation activity of topoisomerase IV by compounds was determined using a gel-based assay format. Compounds were tested over the concentration range 0.001 μM to 100 μM. Final DMSO concentration in the assays was 1% (v/v). One unit of *E. coli* DNA gyrase was incubated with 0.5 μg of relaxed pBR322 DNA at 37°C for 30 minutes under the following conditions: 35 mM Tris.HCl (pH 7.5), 24 mM KCl, 4 mM MgCl2, 2 mM DTT, 1.8 mM Spermidine, 1 mM ATP, 6.5% (w/v) glycerol and 0.1 mg/mL BSA. One unit of *S. aureus* DNA gyrase was incubated with 0.5 μg of relaxed pBR322 DNA at 37°C for 30 minutes under the following conditions: 40 mM HEPES-KOH (pH 7.6)**,** 10 mM magnesium acetate**,** 10 mM DTT**,** 2 mM ATP**,** 500 mM potassium glutamate and 0.05 mg/mL BSA. One unit of *E. coli* topoisomerase IV was incubated with 200 ng kDNA in a 30 μL reaction at 37°C for 30 minutes 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. One unit of *S. aureus* topoisomerase IV was incubated with 200 ng kDNA in a 30 μL reaction at 37°C for 30 minutes under the following conditions: 50 mM Tris.HCl (pH 7.5), 5 mM MgCl2, 5 mM DTT, 1.5 mM ATP*,* 350 mM potassium glutamate and 0.05 mg/mL BSA. One unit of human topoisomerase II was incubated with 200 ng kDNA in a 30 μL reaction at 37°C for 30 minutes under the following conditions: 50 mM Tris HCl (pH 7.5), 125 mM NaCl, 10 mM MgCl2, 5 mM DTT, 0.5 mM EDTA, 0.1 mg/mL BSA and 1 mM ATP. Each reaction was stopped by the addition of chloroform/iso-amyl alcohol (26:1) and Stop Dye (40% sucrose, 100 mM Tris.HCl (pH 7.5), 1 mM EDTA, 0.5 μg/mL bromophenol blue), before being loaded on a 1% TAE gel and electrophoresed. Bands were visualised by ethidium bromide staining, analysed by gel documentation equipment (Syngene, Cambridge, UK) and quantified using Syngene Gene Tools software. Raw data were converted to a percentage of the fully supercoiled control and were analysed using SigmaPlot Version 12.5. Non-linear regression was used to calculate the half-inhibitory concentrations (IC50). All IC50 values were the result of at least two independent experiments. Statistical significance of difference in enzyme inhibition between groups (ciprofloxacin and REDX compounds) and between enzymes (DNA gyrase and topoisomerase IV) was determined using the Student's t-test.

**Antibacterial susceptibility testing**

MICs were determined by the broth microdilution or agar dilution procedure according to the CLSI guidelines.[29](#_ENREF_29) 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 mg/L and a maximum final concentration of 1% DMSO. The agar dilution method involved a two-fold serial dilution of compounds in 24-well plates, giving a typical final compound concentration range of 0.03-128 mg/L and a maximum final solvent 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% CO2. The MIC was determined as the lowest concentration of compound that inhibits visible growth following a 16-24 hour incubation period. Results presented are representative of at least two experiments.

MIC50 and MIC90 determination was performed at IHMA Europe Sàrl (Epalinges, Switzerland) with a selection of recent clinical isolates 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). MICs were performed using frozen 96-well antibacterial panels prepared by broth microdilution in line with CLSI methods M07-A9 and M100-S22,[29](#_ENREF_29), [30](#_ENREF_30) giving a final compound concentration range of 0.004 to 64 mg/L. MIC values were reported as MIC50 and MIC90 for inhibition of 50% and 90% of the isolates, respectively.

**Synergy/antagonism experiments**

Antibacterial combinations were assessed using a two-dimensional checkerboard MIC method.[31](#_ENREF_31) Interpretation of the fractional inhibitory concentration index (FICI) was as described by Odds.[32](#_ENREF_32) Results shown are representative of at least two experiments.

**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 PBS and plated on compound-free CA-MHA. Plates were incubated for up to 48 hours 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). Results shown are representative of at least two experiments.

**Generation of resistant mutants by serial passage**

The generation of resistant bacterial mutants by serial passage was carried out by the broth microdilution method, using the culture representing 0.25 × MIC for the following passage until the desired level of resistance was achieved. At this point, clones were isolated and the MIC confirmed as described previously.

**Whole genome sequencing**

Genomic DNA (gDNA) was extracted from the resistant strains using the EdgeBio PurElute Bacterial Genomic Kit. The gDNA from the *E. coli* strains was purified according to the manufacturer’s instructions while *S. aureus* gDNA purification involved the following modifications: lysostaphin (100 mg/L) and proteinase K (100 mg/L) were incorporated into the spheroplast buffer and Extraction buffer, respectively before incubation at 37°C for 15 min. Purified gDNA was used to create whole genome libraries using NEBNext Ultra kit and 150 bp paired end read sequence data was produced using an Illumina MiSeq at the Next Generation Sequencing facility at the University of Leeds (Leeds, United Kingdom). Read data were stored as FASTQ files and then adaptor sequences where removed using cutadapt software. Data for the wild-type strains were used to construct reference genome sequences using the CLCBio genome assembler. Sequence data for each sample, including the parental control strains, were aligned to the relevant genome using the Burrows-Wheeler Aligner (BWA) software. Variants were identified using VarScan using the appropriate assembled genome as the reference sequence. The resulting data provided a read depth of >100 across the genome. SNPs, insertions and deletions were identified that were prevalent in ≥95% of the reads compared with the parental strains.

**Mammalian 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% CO2. 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 (IC50).

**Results**

**Inhibition of DNA gyrase and topoisomerase IV from *E. coli* and *S. aureus***

The inhibitory effect of REDX04957 and its enantiomers, REDX05967 (S-enantiomer) and REDX05990 (R-enantiomer), on DNA gyrase supercoiling and topoisomerase IV decatenation from *E. coli* and *S. aureus* was investigated using the assays previously described for measuring the anti-gyrase activity of thiazole and isothiazole-based bacterial topoisomerase inhibitors such as isothiazoloquinolone and benzothiazole-containing compounds.[33](#_ENREF_33), [34](#_ENREF_34) Ciprofloxacin was selected as a representative quinolone and tested in parallel for comparison. The data are presented in Table 1.

All the compounds tested, including the quinolone control, inhibited *E. coli* DNA gyrase significantly more potently than *E. coli* topoisomerase IV. REDX05967 showed the most balanced inhibition of the set with an approximate two-fold difference in favour of DNA gyrase and a lower statistically-significant difference of inhibition between the two enzymes compared to ciprofloxacin and the other compounds. REDX04957 and REDX05990 showed an approximate 18-fold and four-fold higher potency, respectively, against DNA gyrase, while ciprofloxacin showed approximately nine-fold higher potency in favour of *E. coli* DNA gyrase (Table 1). REDX04957 and its enantiomers were significantly less active than ciprofloxacin against *E. coli* DNA gyrase (*p* values < 0.01) while REDX05967 and REDX05990 were significantly more potent than the racemate REDX04957 against *E. coli* topoisomerase IV (*p* values < 0.01). All compounds showed a reduced potency against *S. aureus* DNA gyrase compared to *E. coli* gyrase, however only ciprofloxacin and REDX05990 showed a significant difference (*p* values < 0.01). While the quinolone antibiotics show preferential inhibitory activity against topoisomerase IVin *S. aureus*,[35](#_ENREF_35) the Redx compounds showed a balanced or slightly reduced potency against this enzyme compared to DNA gyrase with the exception of the S-enantiomer REDX05967, which showed a significant preference for *S. aureus* topoisomerase IV (*p* value < 0.05) while the R-enantiomer REDX05990 showed a significant preference for *S. aureus* DNA gyrase (*p* value < 0.05).

**Antibacterial susceptibility profile**

The antibacterial susceptibility profile of REDX04957 and its enantiomers compared with ciprofloxacin is shown in Table 2, while Table 3 compares MICs for the wild-type bacterial strains and resistant strains, including those possessing resistance to the quinolones. MICs were within the acceptable range for reference strains and the control antibiotic ciprofloxacin.

MIC testing against a wide range of Gram-negative and Gram-positive bacteria demonstrated a broad-spectrum bactericidal activity for this series similar to that observed for ciprofloxacin (Table 2). The S-enantiomer REDX05967 showed the highest potency against most strains including *A. baumannii, Burkholderia cepacia, K. pneumoniae, Legionella pneumophila, Moraxella catarrhalis, Neisseria gonorrhoeae, N. meningitidis, Stenotrophomonas maltophilia, E. faecalis, S. aureus, S. epidermidis* and *S. pneumoniae*. Notable exceptions were *E. cloacae, E. coli, P. aeruginosa* and *Serratia marcescens* where the racemate compound REDX04957 showed at least equivalent or better activity than either enantiomer. Similar to ciprofloxacin, the bactericidal index of REDX04957 and its two enantiomers was generally in the range 1:1 to 1:4 (Table 2), which is consistent with a cidal mode-of-action. However, unlike quinolone antibiotics, no paradoxical effect was observed with these compounds and the bactericidal effect was maintained at all concentrations above the MBC.[36](#_ENREF_36), [37](#_ENREF_37) To assess whether REDX04957 and its enantiomers are subject to efflux mechanisms, the MICs of compounds were assessed against an efflux pump (AcrA) knockout strain of *E. coli* as well as the isogenic wild-type strain. An efflux ratio of eight-to-16 was observed between the AcrA knockout strain *E. coli* N43 and the wild-type isogenic parent *E. coli* W4573 for REDX04957, REDX05967, REDX05990 and ciprofloxacin demonstrating a similar profile between the compounds (Table 2). In addition, potential mammalian cytotoxicity was evaluated using the HepG2 hepatocellular carcinoma cell line. All three compounds showed IC50 values greater than 128 mg/L.

Further, the activity of REDX04957 and REDX05990 against *E. coli* strains carrying mutations affecting amino acid residues of the quinolone resistance-determining region (QRDR) such as S83L or D87G on the GyrA subunit remained within four-fold of the isogenic parent strain, *E*. *coli* MG1655 (Table 3). For ciprofloxacin and REDX05967, the MIC increased eight-fold with the *E*. *coli* MG1655 GyrA D87G mutant and up to 16-fold in *E. coli* MG1655 GyrA S83L compared to the parent strain (Table 3). Against quinolone-resistant *S. aureus* strains carrying a mutation on the GrlA E84 residue, ciprofloxacin showed a drop-off in antibacterial activity of at least 128-fold compared to the wild type strain *S. aureus* ATCC 29213 with MICs ranging from 32 to greater than 128 mg/L (Table 3). A lower MIC range was obtained against those fluoroquinolone-resistant (FQR) *S. aureus* strains with the isothiazolone compounds (8 - 64 mg/L, Table 3), and most importantly these compounds showed an eight-to-16-fold lower drop off in antibacterial activity than ciprofloxacin. The GrlA V496D mutation in the single mutant *S. aureus* ATCC 29213 QRD10 led to a 32-fold drop off in antibacterial activity of ciprofloxacin whereas a two-fold drop off was observed for REDX04957. The most active compound, REDX05967, was tested against a panel of recent MDR and FQR clinical isolates of Gram-negative bacteria collected between 2012 and 2014. The graphical representations of the MIC distribution against both *E. coli* and *A. baumannii* show a similar profile for both REDX05967 and levofloxacin with two distinct populations of susceptible and resistant isolates with a broader and lower range of MICs for levofloxacin (data not shown). This was reflected in lower MIC50 values than those observed for REDX05967 against both *E. coli* and *A. baumannii*. Against a panel including 25% MDR isolates, REDX05967 and levofloxacin showed a MIC90 of 16 mg/L and 32 mg/L for *A. baumannii*, and 32 mg/L for *E. coli* (Table 4). While the quinolone levofloxacin showed a MIC90 > 64 mg/L against *A. baumannii* panel including 100% MDR isolates, REDX05967 showed a MIC90 of 64 mg/L against the same panel of *A. baumannii* isolates. The opposite result was observed against the panel of MDR *E. coli* isolates.

**Selection of spontaneous resistant mutants to REDX04957 and ciprofloxacin in *E. coli***

The propensity for the development of resistance to REDX04957 in comparison with ciprofloxacin was determined by measuring the spontaneous frequency of resistance. Repeated experiments following exposure of *E. coli* ATCC 25922 to REDX04957 at 4 × and 8 × MIC failed to isolate any mutants. The spontaneous frequency of resistance was calculated to be less than 9.1 × 10-09 at 4 × MIC and less than 2.3 × 10-09 at 8 × MIC. These data are consistent with a balanced dual-targeting mechanism of action. By comparison, the frequency of resistance following exposure to ciprofloxacin was approximately 2.9 × 10-08 at 4 × MIC.

**Selection of resistant mutants to REDX05967 and ciprofloxacin through serial passage**

The CLSI reference strains of *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were used in serial passage experiments as representatives of Gram-negative and Gram-positive bacteria, respectively. Ciprofloxacin was used as a comparator to REDX05967. With *S. aureus* ATCC 29213, resistance to ciprofloxacin (MIC ≥ 4 mg/L)[30](#_ENREF_30) was observed at passage 21, while a greater than 512-fold increase in MIC relative to the starting point was observed at passage 28 (Figure 2). The MIC of REDX05967 increased slightly and remained stable within 16-fold of the original MIC (2 mg/L) up to passage 32, at which stage the experiment was ended. The MIC of ciprofloxacin against *E. coli* ATCC 25922 showed a steady increase over 25 passages to reach a value of 64 mg/L (Figure 2), which corresponds to an increase of more than three orders of magnitude over the original MIC value (0.03 mg/L, Table 1). Ciprofloxacin-resistance was observed at passage 23 (MIC ≥ 4 mg/L).[30](#_ENREF_30) The MIC of REDX05967 showed a slight increase but remained stable at concentration lower than or equal to 1 mg/L up to passage 19 before increasing steadily to reach a maximum of 32 mg/L (256-fold increase) at passage 45, at which stage the experiment was ended. Whole genome sequencing analysis of the REDX05967 serial passage resistant mutants revealed a D87G mutation in the GyrA subunit and a V417A mutation in the ParE subunit at passage 45 in *E. coli*, while no target-specific mutations were observed in *S. aureus* at passage 32 (final passage). A list of mutations identified in both *E. coli* and *S. aureus* serial passages mutants is provided in Table 3.

The MIC value of ciprofloxacin was determined with the REDX05967 serial passage resistant mutants. The serial passage mutants remained susceptible to ciprofloxacin with MIC values lower than or equal to 1 mg/L for both *S. aureus* and *E. coli* REDX05967-resistant mutants (Table 3). The GyrA D87G mutation in the QRDR is commonly observed in Gram-negative quinolone-resistant strains,[38](#_ENREF_38), [39](#_ENREF_39) however, to the best of our knowledge, the ParE V417A mutation observed in the *E. coli* REDX05967-resistant mutant has not been reported in antibiotic-resistant bacterial strains. This genotypic characterisation of REDX05967-resistant mutants combined with the MIC data confirms the absence of cross-resistance between this novel antibiotic class and quinolones.

**Antibacterial interaction of REDX04957 and ciprofloxacin**

The activity of REDX04957 in combination with ciprofloxacin was determined by the checkerboard assay with a selection of ESKAPE pathogens, representing a varied susceptibility profile to ciprofloxacin as shown in Table 2. Synergistic, antagonistic or neutral interactions were determined by calculating the fractional inhibitory concentration index (FICI) and interpreted as described previously.[32](#_ENREF_32) The results of these studies are presented in Table 5. No interaction was observed between ciprofloxacin and REDX04957 for the ciprofloxacin-susceptible and ciprofloxacin-resistant strains of *A. baumannii*, *E. coli*, *K. pneumoniae, P. aeruginosa* and *S. aureus*. In each case the FICI was between 0.75 and 2 (Table 5). The results from these interaction studies indicate that these two inhibitors of the bacterial type II topoisomerases could potentially be used in combination as they show no apparent antagonistic activity.

**Discussion**

The aim of this study was to evaluate the *in vitro* biological profile of a novel class of isothiazolone inhibitors of the bacterial type II topoisomerases as exemplified by REDX04957 and its two enantiomers. Specifically, in order to understand the activity of these compounds at the molecular level and how they might differentiate from the quinolone antibiotics.

The compounds displayed potent activity against both Gram-negative and Gram-positive bacteria, with particular antibacterial activity against quinolone-resistant strains (Table 2). Although the absolute MIC values for REDX04957 and its enantiomers were generally higher than ciprofloxacin against wild-type Gram-positive and Gram-negative bacteria, the relative drop-off in potency for drug-resistant strains compared to the wild-type strains was lower (Table 3). In particular, REDX04957 and its two enantiomers showed increased antibacterial activity compared to ciprofloxacin against *S. aureus* strains with mutations in DNA gyrase and topoisomerase IV that render them resistant to quinolone antibiotics.

MBC assays demonstrated a bactericidal mode-of-action for REDX04957 and its enantiomers, with the MBCs measured as being one-to-four-fold the MIC against all strains tested. This is consistent with the expected mode-of-action for inhibitors of topoisomerases and similar to the quinolones and isothiazoloquinolones.[33](#_ENREF_33)

Similar to other novel bacterial type II topoisomerases inhibitors recently developed, such as the isothiazoloquinolones,[33](#_ENREF_33), [40](#_ENREF_40) these isothiazolone compounds showed good antibacterial activity (MIC < 1 mg/L) against fastidious Gram-negative bacteria including *Haemophilus influenzae, Legionella pneumophila, Moraxella catarrhalis* and *Neisseria* spp. Although the data described here makes no attempt to analyse the structure-activity relationship of this series of isothiazolone compounds and identify the residues associated with anti-gyrase and antibacterial activity, related compounds lacking the isothiazolone ring remain antibacterial, albeit to a lesser extent.[25](#_ENREF_25) The same applies regarding the role of the pyrrolidine, although not essential, the antibacterial activity is enhanced when present. It is also notable that not all thiazole or isothiazole-based compounds display anti-gyrase or antibacterial activity (data not shown); a number of examples inactive or only moderately active in MIC assays have previously been described.[25](#_ENREF_25), [41](#_ENREF_41), [42](#_ENREF_42)

*In vitro* antibacterial activity was also demonstrated for REDX05967 against panels of *A. baumannii* and *E. coli* recent clinical isolates, including 25% of multidrug-resistant strains with MIC50 andMIC90 values of 2 and 16 mg/L, and 0.5 and 32 mg/L, respectively (Table 4). These MIC90 values were as good as those observed for levofloxacin and are similar to those observed for other structurally-similar antibacterial chemotypes previously published.[33](#_ENREF_33) Furthermore, this isothiazolone series showed good selectivity for bacterial cells with no detectable mammalian cytotoxicity up to a concentration of 128 mg/L.

Quinolones are still the most widely-used antibiotics to treat urinary tract infections (UTI) and respiratory tract infections and the rates of quinolone resistance in *E. coli* exceed 50% throughout the world.[43](#_ENREF_43) Likewise, multidrug-resistant *A. baumannii* is a rapidly emerging pathogen associated with high rates of mortality through a number of infections.[44](#_ENREF_44) *A. baumannii* resistance is associated with multiple concomitant mechanisms of resistance such as point mutations on specific cellular targets, a relatively impermeable outer membrane that limits penetration of antibacterials into the cells, and a range of efflux pumps capable of actively removing a broad range of antibacterial agents, including β-lactams, aminoglycosides and quinolones, from the bacterial cell.[45](#_ENREF_45) The quinolone finafloxacin shows improved antibacterial activity over other quinolone antibiotics against quinolone-resistant *A. baumannii* with MIC50 and MIC90 values of 32 mg/L and 64 mg/L, respectively.[46](#_ENREF_46) Interestingly, REDX04957 showed similar MIC50 and MIC90 values (16 mg/L and 64 mg/L, respectively) to finafloxacin against a panel of *A. baumannii* recent clinical isolates including approximately 75% levofloxacin-resistant strains. Thus it would be interesting to investigate further the activity of this non-quinolone antibacterial at a range of relevant physiological pHs for indications such as UTI. In *E. coli*, DNA gyrase has been reported to be the primary target for quinolones.[47](#_ENREF_47) The enzyme inhibition data shown in Table 1 demonstrate the preferential inhibition of DNA gyrase by ciprofloxacin, with a greater than 10-fold higher potency compared to the inhibition of topoisomerase IV. Although REDX04957 showed a similar preference for DNA gyrase with a higher potency against this enzyme, the pure enantiomers REDX05967 and REDX05990 displayed a more balanced inhibition of the bacterial type II topoisomerases.

Unlike the quinolones, which primarily target topoisomerase IV in *S. aureus* leading to first-step mutations in the *grlA* gene in whole-cell assays[48](#_ENREF_48) and confirmed in enzyme inhibition assays in this study (Table 1), a balanced inhibition of DNA gyrase and topoisomerase IV was observed by the three Redx compounds (Table 1). This provides further evidence of a distinct mechanism-of-action between this novel series of bacterial type II topoisomerases inhibitors and the quinolone antibiotics.

The dual-target mechanism-of-action was further supported by the lack of spontaneous resistant mutants isolated at concentrations equivalent to 4 × and 8 × MIC. The FoR in *E. coli* for REDX04957 (<9.1 x 10-09) was approximately one order of magnitude lower than ciprofloxacin and below the expected range (10-06 to 10-09) for a single enzyme-target inhibitor.[10](#_ENREF_10) Mutations conferring high-level of resistance to quinolones usually involve both target and non-target mutations.[17](#_ENREF_17), [49](#_ENREF_49) Therefore, compounds that are not liable to target-specific *de novo* mutations represent a considerable advantage to prevent resistance development. The lack of high-level resistance mutants to REDX05967 was confirmed by serial passage experiments. Generation of resistance to ciprofloxacin required fewer passages than with REDX05967 and only low-level resistant mutants were obtained against this isothiazolone compound for both *S. aureus* and *E. coli* (Figure 2).

No interaction between REDX04957 and ciprofloxacin was observed with the panel of bacterial strains tested. The compatibility between these antibacterial compounds is promising and supports the hypothesis that despite sharing the same targets, REDX04957 and ciprofloxacin exert their inhibitory activity via potentially different molecular interactions with DNA gyrase and topoisomerase IV. This is consistent with the enzyme inhibition and whole-cell data.

In summary, the dual targeting mechanism-of-action distinct from that of the quinolones demonstrates the potential of this novel isothiazolone series as a novel class of antibiotic. Combined with the low potential for resistance development and broad-spectrum bactericidal potency, these findings provide a platform for the development of new non-quinolone antibacterial agents active against the ESKAPE pathogens, including existing and emerging resistant strains.

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**Transparency declarations**

All authors are or have been employees of Redx Anti-Infectives Ltd and may own shares and/or share options in Redx Pharma Plc.

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**Table 1. Inhibition of DNA gyrase supercoiling and topoisomerase IV decatenation by REDX04957 and its enantiomers REDX05967 and REDX05990.** Statistical significance of difference in DNA gyrase and topoisomerase IV inhibition per species was determined using the Student’s t-test where NS = not significant, \* = a *p* value < 0.05, \*\* = a *p* value < 0.01 and \*\*\* = a *p* value < 0.001.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Species | Enzyme |  | IC50 (µM) |  |  |
| Ciprofloxacin | REDX04957 | REDX05967 | REDX05990 |
| *E. coli* | DNA gyrase | 0.72 ± 0.40 | 2.28 ± 0.21 | 4.06 ± 0.40 | 4.45 ± 0.64 |
|  | Topoisomerase IV | 6.15 ± 1.91 | 40.90 ± 1.41 | 9.26 ± 1.11 | 16.20 ± 0.57 |
|  |  | \*\* | \*\*\* | \* | \*\* |
| *S. aureus* | DNA gyrase | 10.75 ± 3.43 | 43.65 ± 32.17 | 27.10 ± 8.63 | 32.70 ± 3.25 |
|  | Topoisomerase IV | 5.33 ± 4.04 | 57.40 ± 14.99 | 19.75 ± 2.33 | 51.45 ± 11.38 |
|  |  | NS | NS | NS | NS |

**Table 2. Antibacterial susceptibility profile of isothiazolones**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Strain | MIC / MBC (mg/L) | | | |
| Ciprofloxacin | REDX04957 | REDX05967 | REDX05990 |
| *Acinetobacter baumannii*  ATCC 19606 | 0.25 / ND | 1 / ND | 0.25 / ND | 2 / ND |
| *Acinetobacter baumannii*  NCTC 13420 | 64 / 64 | 2 / 2 | 1 / 2 | 4 / 8 |
| *Burkholderia cepacia*  ATCC 25416 | 0.5 / ND | 32 / ND | 4 / ND | 16 / ND |
| *Enterobacter cloacae*  NCTC 13406 | 0.015 / 0.25 | 0.06 / 0.12 | 0.12 / 0.12 | 0.5 / 1 |
| *Escherichia coli*  ATCC 25922 | 0.03 / 0.25 | 0.03 / 0.03 | 0.03 / 0.06 | 0.06 / 0.12 |
| *Escherichia coli*  ATCC BAA 2452 | 0.008 / ND | 0.25 / ND | 0.5 / ND | 1 / ND |
| *Escherichia coli*  NCTC 13476 | 64 / >128 | 16 / 16 | 32 / ND | 64 / ND |
| *Escherichia coli*  N43 | 0.004 / ND | 0.03 / ND | 0.0009 / ND | 0.03 / ND |
| *Escherichia coli*  W4573 | 0.03 / ND | 0.25 / ND | 0.015 / ND | 0.5 / ND |
| *Haemophilus influenzae*  ATCC 49247 | 0.008 / ND | 0.25 / 0.25 | 0.5 / 1 | 0.06 / 0.12 |
| *Klebsiella pneumoniae*  ATCC 700603 | 0.25 / 0.25 | 4 / 4 | 4 / 4 | 8 / 16 |
| *Klebsiella pneumoniae*  ATCC BAA 2146 | >128 / >128 | >128 / >128 | >128 / >128 | >128 / >128 |
| *Klebsiella pneumoniae*  NCTC 13439 | 8 / >16 | 64 / >64 | 32 / ND | 64 / ND |
| *Klebsiella pneumoniae*  NCTC 13440 | 1 / ND | 16 / ND | 4 / ND | 16 / ND |
| *Klebsiella pneumoniae*  NCTC 13443 | >128 / >128 | 128 / >128 | >128 / >128 | >128 / >128 |
| *Legionella pneumophila*  ATCC 33152 | 0.03 / ND | 0.03 / ND | 0.015 / ND | 0.06 / ND |
| *Moraxella catarrhalis*  ATCC 25240 | 0.015 / ND | 0.12 / ND | 0.015 / ND | 0.25 / ND |
| *Neisseria gonorrhoeae*  ATCC 49226 | 0.004 / ND | 0.25 / ND | 0.06 / ND | 0.12 / ND |
| *Neisseria gonorrhoeae*  WHO L | 32 / ND | 16 / ND | 16 / ND | 16 / ND |
| *Neisseria meningitidis*  ATCC 13090 | 0.004 / ND | 0.5 / ND | 0.06 / ND | 0.03 / ND |
| *Pseudomonas aeruginosa*  ATCC 27853 | 1 / 2 | 1 / 1 | 1 / 2 | 1 / 4 |
| *Pseudomonas aeruginosa*  NCTC 13437 | 64 / 64 | 64 / >128 | >128 / >128 | >128 / >128 |
| *Serratia marcescens*  ATCC 13880 | 0.06 / ND | 2 / ND | 4 / ND | 16 / ND |
| *Stenotrophomonas maltophilia*  ATCC 13637 | 0.25 / ND | 4 / ND | 2 / ND | 8 / ND |
| *Enterococcus faecalis*  ATCC 29212 | 1 / 2 | 32 / >32 | 16 / ND | 32 / ND |
| *Enterococcus faecium*  ATCC 19434 | 8 / 8 | 128 / >128 | >128 / >128 | 64 / ND |
| *Staphylococcus aureus*  ATCC 29213 | 0.25 / 0.5 | 1 / 2 | 0.5 / 1 | 2 / 2 |
| *Staphylococcus aureus*  NRS 1 | 16 / 64 | 16 / 16 | 8 / ND | 16 / ND |
| *Staphylococcus aureus*  NRS 482 | 16 / 16 | 16 / 16 | 4 / ND | 16 / ND |
| *Staphylococcus aureus*  NRS 74 | >128 / >128 | 4 / ND | 8 / ND | 8 / ND |
| *Staphylococcus epidermidis*  ATCC 12228 | 0.25 / 0.25 | 4 / 16 | 1 / ND | 8 / ND |
| *Streptococcus pneumoniae*  ATCC 49619 | 0.5 / 1 | 16 / >32 | 4 / 4 | 16 / 16 |

ND: not determined

**Table 3. Antibacterial activity of isothiazolones against resistant bacterial strains** **with mutations in DNA gyrase and/or topoisomerase IV**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Strain** | **Target-Specific** | **MIC (mg/L)** | | | |
| **Mutations** | **Ciprofloxacin** | **REDX04957** | **REDX05967** | **REDX05990** |
| *E. coli* MG1655  (Wild-type) |  | 0.008 | 0.5 | 0.06 | 0.25 |
| *E. coli* MG1655 S83L | GyrA S83L | 0.12 | 2 | 1 | 1 |
| *E. coli* MG1655 D87G | GyrA D87G | 0.06 | 2 | 0.5 | 1 |
| *E. coli* EC5967-SP45a | GyrA D87G  ParE V417A | 0.12 | 16 | 8 | 16 |
| *S. aureus* ATCC 29213  (Wild-type) |  | 0.25 | 1 | 0.5 | 2 |
| *S. aureus* ATCC 29213 QRD10 | GrlA V496D | 8 | 2 | ND | ND |
| *S. aureus* ATCC 29213 QRD20 | GrlA E84K  GrlA V496D | 32 | 8 | ND | ND |
| *S. aureus* ATCC 29213 QRF10 | GyrA E88K  GrlA E84K  GrlA V496D | >128 | 64 | ND | ND |
| *S. aureus* SACPX1-SP25 | GyrA S84L  GrlA E84G | 32 | 16 | 8 | 16 |
| *S. aureus* SACPX1-SP28 | GyrA S84L  GrlA E84G  GrlB I473N | 64 | 32 | 4 | 32 |
| *S. aureus* SA5967-SP32b | none | 1 | >16 | 16 | 16 |

ND: not determined

a Mutations identified in *E. coli* EC5967-SP45: Transcription repressor of multidrug efflux (N137T); Membrane protein involved in export (V392G); DNA-directed RNA polymerase beta subunit (R1329I); Transcriptional regulatory protein BasR/PmrA (G94E); Phosphoserine phosphatase (A30V)

b Mutations identified in *S. aureus* SA5967-SP32: Allophanate hydrolase 2 subunit 1 (P128A); Putative metal chaperone involved in Zn homeostasis, GTPase of COG0523 family/cobalamin synthesis protein CobW (I210N)

Note: the SP number corresponds to the serial passage number for the generation of resistant mutants upon exposure to sub-inhibitory concentrations of REDX05967

**Table 4. MIC50 and MIC90 of REDX05967 and levofloxacin for a panel of recent levofloxacin-resistant and multidrug-resistant clinical isolates of *A. baumannii* and *E. coli***

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Compound | Isolates | *A. baumannii* (n) | MIC50 | | MIC90 | *E. coli* (n) | MIC50 | MIC90 |
| (mg/L) | (mg/L) | | (mg/L) | (mg/L) |
| Levofloxacin | All | 45 | 8 | >64 | | 49 | 16 | 32 |
| LVX-R (100%) | 33 | 16 | >64 | | 37 | 16 | 32 |
| MDR (100%) | 23 | 16 | >64 | | 24 | 16 | 64 |
| MDR (25%) | 15 | 0.12 | 32 | | 15 | 0.03 | 32 |
| REDX05967 | All | 45 | 16 | 64 | | 49 | 32 | >64 |
| LVX-R (100%) | 33 | 16 | >64 | | 37 | 32 | >64 |
| MDR (100%) | 23 | 16 | 64 | | 24 | 32 | >64 |
| MDR (25%) | 15 | 2 | 16 | | 15 | 0.5 | 32 |

LVX-R: levofloxacin-resistant, MDR: multidrug-resistant (resistant to at least 6 antibiotics from amikacin, aztreonam, cefepime, ceftazidime, ceftriaxone, colistin, gentamicin, imipenem/ meropenem, levofloxacin, piperacillin-tazobactam and tetracycline).

**Table 5. Interaction of REDX04957 and ciprofloxacin determined by checkerboard assay with a range of representative ESKAPE bacteria**

|  |  |  |  |
| --- | --- | --- | --- |
| Strain | Ciprofloxacin susceptibility\* | FICI | Interaction |
| *A. baumannii* ATCC 19606 | Susceptible | 0.75 | Neutral |
| *A. baumannii* NCTC 13420 | Resistant | 0.75 | Neutral |
| *A. baumannii* ATCC BAA-1710 | Resistant | 1.5 | Neutral |
| *E. coli* ATCC 25922 | Susceptible | 1 | Neutral |
| *E. coli* NCTC 13476 | Resistant | 1 | Neutral |
| *K. pneumoniae* NCTC 13440 | Susceptible | 0.75 | Neutral |
| *K. pneumoniae* NCTC 13439 | Resistant | 0.75 | Neutral |
| *P. aeruginosa* ATCC 27853 | Susceptible | 2 | Neutral |
| *P. aeruginosa* NCTC 13437 | Resistant | 1 | Neutral |
| *S. aureus* ATCC 29213 | Susceptible | 1.5 | Neutral |

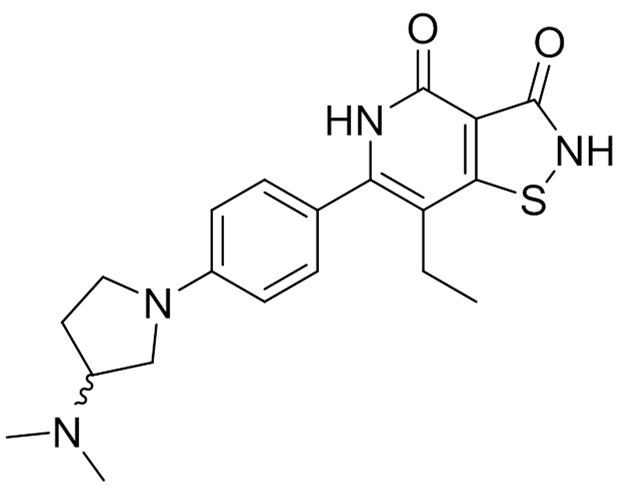
FICI: Fractional Inhibitory Concentration Index; \* Ciprofloxacin susceptibility breakpoints based on CLSI document M100-S22 (R: MIC ≥ 4 mg/L, S: MIC ≤ 1 mg/L)

**FIGURE LEGENDS**

Figure 1. Chemical structure of REDX04957.

Figure 2. Isolation of ciprofloxacin- and REDX05967- resistant mutants of *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 by serial passage.

**Figure 1**



**Figure 2**

