De Novo Design of Type II Topoisomerase Inhibitors as Potential Antimicrobial Agents Targeting a Novel Binding Region

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Abstract

By 2050 it is predicted that antimicrobial resistance will be responsible for 10 million global deaths annually, more deaths than cancer, costing the world economy \$100 trillion.¹ Clearly, strategies to address this problem are essential as bacterial evolution is rendering our current antibiotics ineffective. The discovery of an allosteric binding site on the established antibacterial target DNA gyrase offers a new medicinal chemistry strategy. As this site is distinct from the fluoroquinolone binding site, resistance is not yet documented. Using *in silico* molecular design methods, we have designed and synthesised a novel series of biphenyl-based inhibitors inspired by a published thiophene-based allosteric inhibitor. This series was evaluated *in vitro* against *Escherichia coli* DNA gyrase and *E. coli* topoisomerase IV, exhibiting IC₅₀ values in the low micromolar range for gyrase and yielding a uniquely equipotent inhibition of gyrase and topo IV. The

promising structure-activity relationships reported herein suggest insights to further exploit this allosteric site, offering a pathway to overcome developing fluoroquinolone resistance.

Keywords: DNA gyrase, topoisomerase IV, antimicrobial resistance, structure-based drug design, *de novo* design, allosteric inhibitors

The evolution of antibiotic resistance poses an enormous threat to human health. The discovery of penicillin in 1928 heralded the beginning of the antibiotic era, revolutionising the treatment of bacterial infections. Antibiotics then became a staple in modern medical procedures such as surgery and organ transplantation. However, following the golden period of antimicrobial drug discovery between the 1960s and 1980s, a decline in novel antibiotic FDA approval coupled with a rise in antimicrobial resistance (AMR), has led to a growing increase in the number of untreatable bacterial infections.²

DNA gyrase and topoisomerase IV are essential bacterial type II topoisomerases that control DNA topology during DNA replication, transcription and other DNA-associated processes.^{3, 4} DNA gyrase introduces negative supercoils into bacterial DNA *via* an ATP-dependent mechanism, whereas topoisomerase IV primarily eliminates DNA entanglements that occur during DNA replication. Both enzymes are composed of two proteins, coded for by the *gyrA* and *gyrB* genes for DNA gyrase, and the *parC* and *parE* genes in the case of topoisomerase IV.^{5, 6} These two protein assemblies are composed of four protein subunits, forming heterotetrameric complexes: A₂B₂ for DNA gyrase, and C₂E₂ for topoisomerase IV. They are well-documented targets for antimicrobial therapy, with the fluoroquinolones being renowned for possessing a “dual-targeting” mechanism with the possibility of inhibiting both enzymes simultaneously. Dual-targeting is an attractive prospect for antimicrobial drug discovery, as inhibition of two enzymes simultaneously presents bacteria with a significant challenge towards resistance evolution.^{7, 8}

DNA gyrase and topoisomerase IV possess a high degree of sequence and structural similarity, which favours the dual-targeting approach. They possess limited sequence similarity to that of human topoisomerase II which allows for the design of selective inhibitors for these bacterial topoisomerase enzymes over the human topoisomerase II.⁹

The more established sites on the topoisomerases for the interaction of inhibitors are associated with their DNA- and ATP-binding sites.^{6, 10} In the case of the fluoroquinolones, these drugs bind to the enzyme-DNA complex and effectively “trap” the bound DNA within the enzyme by forming key interactions within the DNA-binding site *via* a water-metal ion bridge.¹¹ However, despite the success of dual-targeting agents such as the fluoroquinolones (FQs), as well as the relatively slow rate at which bacterial resistance to these drugs has occurred, resistance within the clinic is growing.¹² There are concerns that eventually, these antibacterial drugs may become ineffective.

Resistance to fluoroquinolones has developed largely due to point mutations developing in nature and have led to an increasing resistance to fluoroquinolones in recent decades. Point mutations within the *gyrA* (e.g. S81, S83, D87 and E85; *Escherichia coli* numbering) and *gyrB* (e.g. N426 and K447) genes of DNA gyrase, as well as the *parC* (e.g. S79, D83 and E84) and *parE* (e.g. D435) genes of topoisomerase IV contribute to said antimicrobial resistance.¹² The discovery of potent and novel antimicrobial agents that either bind to alternative regions within the topoisomerases or operate *via* different mechanisms is therefore paramount in combatting the rise of bacterial resistance.

Allosteric binding sites offer promising, alternative mechanistic types of enzyme inhibition. Chan *et al.* reported one such example within a *Staphylococcus aureus* DNA gyrase structure containing a thiophene-carboxamide inhibitor (**1**).^{13, 14} Inspection of the co-crystal structure revealed the inhibitor to be bound within a pocket between the GyrA and GyrB subunits. This region is remote from the fluoroquinolone binding site. Inhibitor **1** (IC₅₀: 0.30 µM, *E. coli* gyrase) adopts a conformation within this pocket involving the formation of key interactions with residues R630 and E634 (*S. aureus* numbering), with the amide carbonyl of the inhibitor acting as a hydrogen bond acceptor for the neighbouring arginine residue, and the terminal amine of the ligand acting as a hydrogen bond donor for the neighbouring glutamate residue. It is also theorised that a key interaction forms between the amide hydrogen bond donor and a water molecule trapped within the X-ray co-crystal structure, forming a water-mediated hydrogen bond to P1343 (Figure 1). The compound inhibits the supercoiling ability of gyrase, as well as stabilising gyrase-dependent DNA cleavage, but does not inhibit topoisomerase IV (IC₅₀: >540 µM), questioning whether dual-targeting can be achieved within this allosteric site.¹³ Ultimately,

the development of inhibitor **1** was terminated due to observed *in vivo* toxicity issues, although a later publication by the same group described further examples of fused heterocycles replacing or incorporating the thiophene.¹⁴

Figure 1. (A) Inhibitor **1** (gold) within the allosteric site of *S. aureus* DNA gyrase (5NPP)¹³. Key hydrogen bonding interactions are shown as yellow dashes to DNA gyrase residues (purple and labelled). Note, the water-mediated hydrogen bond between the amide NH and P1343. (B) 2D schematic of panel A, highlighting the conserved water in red.

The principals of structure-based ligand design were introduced in the 1980s.¹⁵ Following significant developments in technological processing power, *in silico* software, and the availability of high-resolution crystal structures, alternative computational methods to high-throughput screening (HTS) became commonplace to investigate protein target sites and initiate drug discovery projects. SPROUT¹⁶ is a program used for *de novo* structure-based molecular design, using a fragment-based approach to design novel scaffolds that can then be ranked by predicted binding affinity. It has been used to design inhibitors of several enzymes derived from infectious diseases.¹⁷⁻²¹ Using the co-crystal structure of *S. aureus* DNA gyrase with thiophene inhibitor **1** (PDB ID: 5NPP)¹³, we aimed to design novel compounds that bind within the allosteric site and may offer hope in combating the threat of fluoroquinolone resistance by retaining gyrase activity against FQ-resistant strains and simultaneously possessing activity against topoisomerase IV.

Based on the X-ray conformation of compound **1** from 5NPP, we decided to utilise the three key hydrogen bonding interactions in our molecular designs (R630, E634 and the

water-mediated bond to P1343). This allowed the ‘right-hand’ portion of inhibitor **1** (as illustrated in Figure 1) to be kept consistent within the SPROUT software, and the hydrophobic left-hand portion to be varied by the steric constraints of the binding cavity. These constraints vary between gyrase and topo IV, but the sequence conservation of the allosteric site is high amongst pathogenic bacterial species and topoisomerase enzymes, validating 5NPP as a model system.¹³ Amongst the initial results, a biphenyl molecular scaffold, as represented by biphenyl inhibitor **2**, was a synthetically attractive candidate to test the potential of our *de novo* design approach to targeting this binding site. In summary, compound **2** was designed to retain the same hydrogen bonding interactions as observed for thiophene inhibitor **1**, but the biphenyl portion was predicted to make an additional favourable interaction with R1342 of gyrase *via* a cation- π interaction (Figure 2).

Figure 2. (A) Novel biphenyl inhibitor design **2** (gold) modelled within the allosteric site of *S. aureus* DNA gyrase (5NPP)¹³. Key hydrogen bonding interactions shown in yellow and key residues (purple) are labelled. (B) 2D schematic of panel A.

Biphenyl inhibitor **2** was synthesised and tested in an *in vitro* *E. coli* DNA gyrase supercoiling assay, revealing it to be a moderate inhibitor of *E. coli* gyrase (IC₅₀: 60 μ M, chemical synthesis and biochemical assays described below). With this encouraging result in mind, we endeavoured to explore the structure-activity relationships (SARs) by varying the substitution pattern on the biaryl unit.

To help guide the design of these substituted analogues, the docking module Glide within the Schrödinger Maestro software package²² was used. Various functional groups were computationally added to the biphenyl ring system, and these compounds were then docked within the allosteric site of the 5NPP co-crystal structure to explore the steric and electronic characteristics within the hydrophobic cavity. Compounds containing electron-donating groups on the terminal biaryl ring (Table 1), such as 2-methoxy (**3**) and 3-ethoxy (**4**), were predicted to display enhanced binding to the enzyme through higher docking scores (an empirical measure of predicted binding affinity). This was rationalised as increasing the magnitude of the cation- π interaction due to enhanced electronic attraction between the inhibitor and R1342.

Certain substituents were predicted to induce a twist in the structure, akin to the docked pose of biphenyl **2** (Figure 2), through with aspirations to increase the torsion angle between the two phenyl rings. This ring twist was also observed in the 5NPP structure containing the thiophene inhibitor (**1**), and therefore was likely tolerated or required for bioactivity. The ring twist was calculated to be 43° in the case of thiophane compound **1**, and 61° for unsubstituted biphenyl compound **2**. Additionally, several other substituted molecules, containing strong electron-withdrawing substituents (inhibitors **5**, **11**, **12**, **13** and **14**), halogen atoms (inhibitors **9** and **10**), and combinations of some of these substituents (inhibitors **6** and **7**) were also prepared to explore the SARs *in vitro* within this inhibitor series. A phenyl-lacking derivative (**15**) was synthesised to begin explorations into the potential hydrophobic binding requirements of the biphenyl inhibitors.

Compound	R	IC ₅₀ (μM)	Compound	R	IC ₅₀ (μM)
2		60	9		35
3		20	10		39
4		20	11		73
5		21 ± 2.5	12		42
6		12 ± 2.1	13		24 ± 2.2

7		17 ± 1.9
8		46
14		41 ± 7.0
15	Br	>200

Table 1. *In vitro* data for a novel biphenyl series of DNA gyrase inhibitors designed to investigate SARs and cation- π potential within the *E. coli* DNA gyrase allosteric site. IC₅₀ results for inhibition of gyrase supercoiling are the mean $\pm$ SD (n=3). Where error values are not determined, n=2.

The synthesis of these compounds was readily achieved (Scheme 1). The commercially available (*S*)-2-amino-1-phenylethanol starting material (**16**) underwent Boc-protection to result in **17** in excellent yield. This was ahead of a Mitsunobu reaction in the form of a Gabriel synthesis to switch the alcohol for a phthalimide functionality, leading to inversion of stereochemistry simultaneously. This resulted in compound **18** in good yield. This was subsequently followed by cleavage of the phthalimide unit using hydrazine to result in chiral primary amine **19** in good yield. Amine **19** was then coupled to 3-bromobenzoyl chloride to form the key amide linker, with the 3-bromo position of **20** primed for the ensuing Suzuki-Miyaura coupling of various substituted boronic acids. Intermediate **20** was again formed in good yield. The Suzuki-Miyaura couplings proved facile but proceeded in relatively poor yields. The Boc-protecting group was then removed using strong acid in the form of 4 N HCl in dioxane to give the final biphenyl inhibitors **2-15** in excellent yield.

Scheme 1. Reagents and conditions: a) Boc_2O , THF, RT, 1 hour, 89%. b) Phthalimide, PPh_3 , DEAD, THF, N_2 , 0°C – RT, 18 hours, 63%. c) $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$, EtOH, 60°C , 4 hours, 74%. d) 3-Bromobenzoyl chloride, NEt_3 , DCM, N_2 , RT, 22 hours, 77%. e) Substituted boronic acid, $\text{Pd}(\text{PPh}_3)_4$, propanol, 2 M Na_2CO_3 aqueous solution, N_2 , reflux, 20-35%. f) 4 N HCl in dioxane, RT, 65-93%.

The compounds were tested in a DNA supercoiling assay with *E. coli* DNA gyrase using gel electrophoresis. This is a semi-quantitative assay which is used to identify potent compounds relative to a positive control (usually ciprofloxacin, IC_{50} : $0.3\ \mu\text{M}$, *E. coli* gyrase¹³) and establish the SARs within a compound series. Compounds were tested in duplicate ($n=2$) or triplicate ($n=3$) and standard errors calculated (Table 3S supplementary information).

Pleasingly, as predicted, compounds containing electron-donating substituents (**3**, **4**, **6**, **7**, and **8**) showed enhanced inhibitory potency versus the unsubstituted system (**2**, IC_{50} : $60\ \mu\text{M}$), with the optimum inhibitor **6** displaying a 5-fold increase in binding affinity against gyrase (IC_{50} : $12 \pm 2.1\ \mu\text{M}$). Analysis of the biological data for compounds **9-14** revealed that derivatisation of the biphenyl terminal ring is generally tolerated, with moderate increases in activity being witnessed compared to unsubstituted biphenyl **2**. The incorporation of strong electron-withdrawing substituents such as 2-trifluoromethyl (**5**) and 3-nitro (**11**) led to an increase in observed affinity for the former (IC_{50} : $21 \pm 2.5\ \mu\text{M}$), but a decrease for the latter (IC_{50} : $73\ \mu\text{M}$), compared to unsubstituted biphenyl **2**. This binding

affinity increase was also observed for the two ester derivatives synthesised in **13** (IC_{50} : $24 \pm 2.2 \mu M$) and **14** (IC_{50} : $41 \pm 7.0 \mu M$).

Generally, we observed no significant difference in activity between compounds with similar substituents at the *ortho* or *meta* positions. Notably, when the terminal biphenyl ring was replaced with a bromine atom (**15**), activity dropped at least 3-fold compared to phenyl-containing inhibitor **2**. Compound **15** (IC_{50} : $>200 \mu M$), is predicted to retain the hydrogen bonding network *in silico* observed for all biaryl analogues, alluding to the importance of the hydrophobic contributions to binding affinity for this compound series.

With a broad SAR profile in hand, further analogues were synthesised that would probe the hydrophobic and steric requirements of the hydrophobic cavity in DNA gyrase, investigate the chirality at the asymmetric centre and explore the addition of substituents to the chiral phenyl ring. The methyl and chloro substituents on thiophene inhibitor **1** appeared to optimally fill small cavities within hydrophobic pocket. We attempted to explore this on our biaryl series by introducing methyl groups at the 4-position on the benzamide ring and at the 5-position on the terminal phenyl respectively, using the synthetic steps outlined in scheme 1 (compounds **21-23**, Table 1S supplementary information). These changes proved detrimental to activity in the case of compound **21** (IC_{50} : $153 \pm 2.5 \mu M$), while compounds **22** (IC_{50} : $63 \pm 5.2 \mu M$), and **23** (IC_{50} : $56 \pm 2.1 \mu M$), displayed similar activity to that of unsubstituted biphenyl inhibitor **2**. It was concluded that vector growth into the hydrophobic pocket should more closely mimic that of compound **1**.

The (*R*)-chirality present in thiophene **1** was required for binding to the allosteric site by positioning the chiral phenyl moiety in a favourable vector. The (*S*)-isomer of **1** was reported as being ~30-fold less active.¹³ In order to ascertain whether the *R*-chirality was crucial for our biphenyl inhibitor series, a number of (*S*)-chiral compounds (**24-26**) were prepared using an analogous sequence to that shown in Scheme 1 but conversely beginning from (*R*)-2-amino-1-phenylethanol (Table 2). As expected, these 'chirally-reversed' compounds showed a general decrease in activity compared to their enantiomeric (*R*)-counterparts: **24** (IC_{50} : $76 \mu M$) vs. **2** (IC_{50} : $60 \mu M$), **25** (IC_{50} : $36 \mu M$) vs.

3 (IC₅₀: 20 μM), and **26** (IC₅₀: 180 μM) vs. **4** (IC₅₀: 20 μM), although the fold-decrease was only significant for compound **26** (9-fold).

Compound	R ₁	R ₂	IC ₅₀ (μM)
24			76
25			36
26			180
27			41
28			45
29			41
30			62

31		>200
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Table 2. Further SAR exploring the role of chirality and substitution on the phenyl ring. IC₅₀ values were determined against *E. coli* DNA gyrase in a supercoiling assay.

To explore the role of substitution on the (*R*)-phenyl moiety within the compound series, several analogues were synthesised where the phenyl ring was replaced by a phenol. This ring system was selected due to favourable molecular modelling predictions which suggested that this solvent-exposed region of the pocket could potentially offer an additional bonding interaction. We aspired to form said supplementary hydrogen bond to R630 *via* an intermolecular bridge with a labile water molecule. For these analogues, an optically pure starting material was not commercially available, and so racemic starting materials were used during their synthesis (see Scheme 1S in supplementary material for adapted synthetic route). Five analogues (**27-31**) were synthesised and tested *in vitro*, with all being moderate inhibitors of DNA gyrase, demonstrating the scope for further derivatisation in this region *via* the chiral phenyl ring.

Finally, several biphenyl inhibitors underwent extensive evaluation *in vitro* against *E. coli* topoisomerase IV and *E. coli* and *S. aureus* bacterial cells, for which IC₅₀ and MIC values were determined respectively (Table 3). DNA relaxation assay with *E. coli* topo IV is a semi-quantitative assay used to identify potent compounds relative to a positive control (novobiocin, IC₅₀: 11 µM, *E. coli* topo IV). Compounds were tested in duplicate (n=2) or triplicate (n=3) and standard errors calculated.

Compound	R	Ring Chirality	<i>E. coli</i> DNA Gyrase IC ₅₀ (μM)	<i>E. coli</i> Topo IV IC ₅₀ (μM)	<i>E. coli</i> MIC (μg/mL)	<i>S. aureus</i> MIC (μg/mL)
5		(R)	21 ± 2.5	>100	>64	32
6		(R)	12 ± 2.1	>100	>64	64
7		(R)	17 ± 1.9	64 ± 2.0	>64	32
11		(R)	73	94	>64	>64
26		(S)	180	>100	>64	64
27		Racemic (3-OH)	41	>100	>64	>64

Table 3. Biphenyl inhibitors with further biological evaluation reported. IC₅₀ results are the mean $\pm$ SD (n=3). Where error values are not determined, n=2. **7** indicates the most equipotent gyrase and topo IV inhibitor discovered to-date.

This further biological evaluation yielded exciting results, where topoisomerase IV inhibition was observed for compounds **7** and **11** (Table 3).

Notably, **7** offers significantly more *E. coli* topo IV activity than that of thiophene inhibitor **1** developed by Chan *et al.* (**1**, topo IV IC₅₀: >540 μ M), though DNA gyrase inhibition remains lower when compared to said thiophene inhibitor (gyrase IC₅₀: 0.3 μ M for **1**). The best inhibitor from the subsequent fused heterocyclic work performed by the same group resulted in a more potent gyrase inhibitor (gyrase IC₅₀: 0.16 μ M) and an improvement in topoisomerase IV activity (topo IV IC₅₀: ~90 μ M).¹⁴ When compared to the best inhibitor reported by Thalji *et al.*, **7** is ca. 1.5-fold more potent against topo IV but is not as potent against gyrase.

Biphenyl inhibitor **7** is ca. 4-fold more potent against gyrase than topo IV, but crucially demonstrates good activity against both enzymes, something not achieved by the thiophene inhibitor (**1**). This level of near equipotency holds great potential for the dual-targeting ability of the allosteric site, as our allosteric inhibitors start to mirror the activity trend of the FQ antibiotics (ciprofloxacin, gyrase IC₅₀: 0.6 μ M, topo IV IC₅₀: 5.7 μ M).¹³ It is essential to remember that bacterial resistance has yet to evolve within the allosteric site, and as such holds great promise in designing the next generation of gyrase and topo IV inhibitors.

Biphenyl inhibitors **5** and **7** were also found to possess some Gram-positive cellular activity vs. *S. aureus* (MIC: 32 μ g/mL). The most potent DNA gyrase biphenyl inhibitor (**6**) also records some bactericidal activity against *S. aureus* (MIC: 64 μ g/mL). None of the biphenyl inhibitors assessed displayed cellular activity against *E. coli* (MIC: >64 μ g/mL) despite largely adhering to the eNTRY rules for accumulation into Gram-negative bacteria.²³

In summary, the use of a *de novo* molecular design approach has successfully led to the identification of a new series of biphenyl-based inhibitors targeting a novel allosteric binding site within bacterial DNA gyrase. Numerous compounds were synthesised based

on molecular modelling hypotheses and subsequently tested *in vitro* against DNA gyrase using a supercoiling assay. This led to the development of biphenyl inhibitor **6** which had an improved IC₅₀ value of $12 \pm 2.1 \mu\text{M}$ against DNA gyrase compared to our initial parent biphenyl compound **2** (gyrase IC₅₀: $60 \mu\text{M}$). Synthesis of biphenyl inhibitor **7** (gyrase IC₅₀: $17 \pm 1.9 \mu\text{M}$) reported equipotent gyrase inhibition to that of inhibitor **6**, but more importantly displayed inhibition of *E. coli* topo IV (topo IV IC₅₀: $64 \pm 2.0 \mu\text{M}$), a feature not observed with the thiophene compound (**1**).

Further work is underway to improve both the gyrase and topo IV activity of the biphenyl series, as well as the development of alternative scaffolds for this allosteric site which may display comparable dual gyrase and topo IV activity. Our approach demonstrates that a combination of traditional medicinal chemistry and *in silico* molecular modelling can predict bioactive trends for this target and may lay the foundations to identify future novel gyrase and topo IV antibacterial agents.

Author contributions KMO and MJM designed the compounds. KMO, JFN, BKLB and HLJ synthesised the compounds. LF, TG and LRA evaluated the compounds. MJM, AM and CWGF designed the study. KMO, MJM, LF, AM and CWGF wrote the manuscript. All authors contributed to the editing of the manuscript.

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