Structure-guided design of antibacterials that allosterically inhibit DNA gyrase

Reema K. Thalji, Kaushik Raha, Daniele Andreotti, Anna Checchia, Haifeng Cui, Giovanni Meneghelli, Roberto Profeta, Federica Tonelli, Simona Tommasi, Tania Bakshi, Brian T. Donovan, Alison Howells, Shruti Jain, Christopher Nixon, Geoffrey Quinque, Lynn McCloskey, Benjamin D. Bax, Margaret Neu, Pan F. Chan, and Robert A. Stavenger

A series of DNA gyrase inhibitors were designed based on the X-ray structure of a parent thiophene scaffold with the objective to improve biochemical and whole-cell antibacterial activity, while reducing cardiac ion channel activity. The binding mode and overall design hypothesis of one series was confirmed with a co-crystal structure with DNA gyrase. Although some analogs retained both biochemical activity and whole-cell antibacterial activity, we were unable to significantly improve the activity of the series and analogs retained activity against the cardiac ion channels, therefore we stopped optimization efforts.

Multi-drug resistant (MDR) bacteria, especially MDR Gram-negative bacteria, are a rising public health threat. Without effective antibiotic therapy, many mainstays of modern medicine such as chemotherapy and major surgery would not be possible. This high unmet medical need has been noted by societies, governments, and even the United Nations. Due to this, the need to discover and develop novel treatments for resistant bacterial infections is higher than ever. To address this need, new ways of working are required. One example of this is the Innovative Medicines Initiative’s ENABLE project, whose aim is to optimize hits and leads which target Gram-negative bacteria in semi-open innovation, consortium format. As part of the ENABLE project, we recently reported a series of thiophene-based antibacterials which target the bacterial topoisomerase DNA gyrase via binding to a previously unidentified allosteric site on the enzyme. The bacterial topoisomerases DNA gyrase and topoisomerase IV are essential enzymes which control DNA topology and are the targets of the clinically validated fluorquinolone antibiotics, in addition to several other classes reported in the literature or in clinical development.

The thiophene-based antibacterial 1 (Figure 1) is an inhibitor of bacterial DNA gyrase with modest antibacterial activity against a range of Gram-negative bacterial pathogens. Unlike most other inhibitors of bacterial topoisomerases, the lead compound 1 only showed significant activity against DNA gyrase with essentially no activity against the related enzyme topoisomerase IV (IC₅₀ >500 μM) in a decatenation of kinetoplast DNA inhibition assay. As part of an overall medicinal chemistry program on this series, we sought to modify the core of these inhibitors. Examination of the binding mode of 1 to Staphylococcus aureus (S. aureus) DNA gyrase (Figure 3A) suggested that two cyclization strategies to bicyclic templates could be tolerated (Figure 1). Ring closure route a retains the thiophene core and fuses a 6-ring heterocycle, while ring closure route b changes the thiophene to a 6-membered benzo- or heterocycle and fuses a 5- or 6-membered heterocycle. We hypothesized that appropriately constraining the core might improve biochemical activity at DNA gyrase; here we report our results on the synthesis and evaluation of exemplars of both cyclization approaches (Figure 2). The gyrase inhibitors 2 through 12 (Figure 2) were synthesized as described in Schemes 1-7. The synthesis of thieno[3,2-d]pyrimidin-4(3H)-one core inhibitor 2, derived from ring closure route a (Figure 1), started with commercially available thiophene and proceeded via Suzuki coupling followed by saponification to provide the corresponding carboxylic acid. PyBOP-mediated coupling with the protected (S)-phenylethylenediamine provided 14. Heating in the presence of trimethoxymethane and acetic anhydride provided the cyclized product which was deprotected with trifluoroacetic acid to give compound 2.

ABSTRACT

A series of DNA gyrase inhibitors were designed based on the X-ray structure of a parent thiophene scaffold with the objective to improve biochemical and whole-cell antibacterial activity, while reducing cardiac ion channel activity. The binding mode and overall design hypothesis of one series was confirmed with a co-crystal structure with DNA gyrase. Although some analogs retained both biochemical activity and whole-cell antibacterial activity, we were unable to significantly improve the activity of the series and analogs retained activity against the cardiac ion channels, therefore we stopped optimization efforts.
Figure 1. Gyrase inhibitor 1 and new bicyclic designs.

Figure 2. DNA gyrase inhibitors disclosed in this paper.

Scheme 1. Reagents and conditions: (a) 2-chlorobenzeneboronic acid, K$_2$CO$_3$, Pd(PPh$_3$)$_4$, dioxane, H$_2$O, 100 ºC, 98%; (b) LiOH, MeOH, H$_2$O, 86%; (c) N-Boc-((S)-phenylethylenediamine, iPr$_2$NEt, PyBOP, MeOH, 67%; (d) trimethoxymethane, Ac$_2$O, 100 ºC, 25%; (e) TFA, CH$_2$Cl$_2$, 61%.

The synthesis of 3 and derivatives 11 and 12 is described in Scheme 2. Arylation of bromide 15 provided 16 followed by formation of the N-hydroxy amide under basic conditions followed by cyclization with carbonyldiimidazole provided the key benzisoxazolone intermediate 17. Treatment with alcohol 18 under Mitsunobu conditions afforded the desired O-alkylated product 21. Formation of the HCl salt afforded target compound 3. Alternatively, reaction of 21 with 1H-pyrazole-1-carboximidamide followed by HCl salt formation provided the guanidine analog 12. In an analogous manner, intermediate 17 was coupled with Boc-protected 2-amino-1-(pyridin-2-yl)ethanol (19), followed by deprotection and salt formation to give target 11.

Scheme 2. Reagents and conditions: (a) 2-chlorobenzeneboronic acid, Cs$_2$CO$_3$, Pd(PPh$_3$)$_4$, dioxane, H$_2$O, 100 ºC, 98%; (b) NH$_2$OH, MeOH, 0 ºC; 2) LiAlH$_4$, THF, 40 ºC, Boc$_2$O, NaHCO$_3$, THF/H$_2$O (5% over 3 steps). The synthesis of O-alkylated indazoles 4 and 5 proceeded with a similar route and is shown in Scheme 3, starting with Suzuki coupling and methyl ester formation to give 24. Displacement of the aryl fluoride with hydrazine at 100 ºC, followed by protection/activation with Boc-anhydride and treatment with a strong base gave the indazolanone core 25. Mitsunobu reaction with alcohol 18 followed by removal of the Boc group with trifluoracetic acid and swapping to the HCl salt gave target compound 4. Selective protection of the primary amine of 4 with Boc-anhydride followed by methylation of the indazole nitrogen with iodomethane, deprotection, and salt switching gave compound 5.

Scheme 3. Reagents and conditions: (a) 2-chlorobenzeneboronic acid, Cs$_2$CO$_3$, Pd(PPh$_3$)$_4$, dioxane, H$_2$O, 100 ºC, 91%; (b) SOCl$_2$, tol, MeOH, 92%; (c) hydrazine, MeOH, 100 ºC, 75%; (d) Boc$_2$O, iPr$_2$NEt, DMAP, CH$_2$Cl$_2$; (e) aq. NaOH, 71% over 2 steps; (f) 19, PPh$_3$, DIAD, THF, rt, 73%; (g) TFA, CH$_2$Cl$_2$; (h) HCl, Et$_2$O, 67% over 2 steps; (i) Boc$_2$O, NaHCO$_3$, THF, 0 ºC; (j) Me$_3$S, K$_2$CO$_3$, DMF; (k) TFA, CH$_2$Cl$_2$; (l) HCl, Et$_2$O, 33% over 4 steps from 5.

The synthesis of the N-alkylated indazoles 6 and 7 started with arylation of 5-bromo-indazole (26) followed by iodination in the presence of a strong base to give compound 27 (Scheme 4). Blocking the N1-H upon reaction with either Boc-anhydride or iodomethane provided compounds 28 and 29, respectively, which were each aminated using palladium catalysis and benzophenone imine followed by treatment with hydroxylamine to give the 3-amino analogs 30 and 31, respectively. Heating with nitrostyrene provided the 1,4-addition products which were reduced with nickel boride in cold THF/methanol. Trifluoroacetic acid deprotection of the N-Boc group of 30 and formation of the HCl salt provided target compound 6. Target 7 was made similarly from 31 by simple formation of the HCl salt following reduction of the nitrostyrene adduct.

Scheme 4. Reagents and conditions: (a) 2-chlorobenzeneboronic acid, Cs$_2$CO$_3$, Pd(PPh$_3$)$_4$, dioxane, H$_2$O, 100 ºC, 91%; (b) SOCl$_2$, tol, MeOH, 92%; (c) hydrazine, MeOH, 100 ºC, 75%; (d) Boc$_2$O, iPr$_2$NEt, DMAP, CH$_2$Cl$_2$; (e) aq. NaOH, 71% over 2 steps; (f) 19, PPh$_3$, DIAD, THF, rt, 73%; (g) TFA, CH$_2$Cl$_2$; (h) HCl, Et$_2$O, 67% over 2 steps; (i) Boc$_2$O, NaHCO$_3$, THF, 0 ºC; (j) Me$_3$S, K$_2$CO$_3$, DMF; (k) TFA, CH$_2$Cl$_2$; (l) HCl, Et$_2$O, 33% over 4 steps from 5.

The synthesis of O-alkylated indazoles 4 and 5 proceeded with a similar route and is shown in Scheme 3, starting with Suzuki coupling and methyl ester formation to give 24. Displacement of the aryl fluoride with hydrazine at 100 ºC, followed by protection/activation with Boc-anhydride and treatment with a strong base gave the indazolanone core 25. Mitsunobu reaction with alcohol 18 followed by removal of the Boc group with trifluoracetic acid and swapping to the HCl salt gave target compound 4. Selective protection of the primary amine of 4 with Boc-anhydride followed by methylation of the indazole nitrogen with iodomethane, deprotection, and salt switching gave compound 5.
Scheme 4. Reagents and conditions: (a) 2-chlorobenzeneboronic acid, Cs₂CO₃, Pd(PPh₃)₄, dioxane, H₂O, 160 ºC, 70%; (b) Me, K₂CO₃, DMF, 90%; (c) benzophenone imine, Na₂(dba)₃, xantophos, Cs₂CO₃, dioxane, 100 ºC; (d) NiCl₂, NaBH₄, MeOH, THF, -20 ºC; (e) TFA, CH₂Cl₂; (f) HCl, Et₂O.

The synthesis of isoquinoline 8 started with SsAr reaction of compound 32 with mono-protected diamine 33 (Scheme 5). Suzuki coupling with 2-chlorobenzeneboronic acid provided compound 34 followed by deprotection of the N-Boc group, and formation of the HCl salt gave target compound 8.

Scheme 5. Reagents and conditions: (a) Pyridine, 145 ºC microwave; (b) 2-chlorobenzeneboronic acid, Cs₂CO₃, Pd(PPh₃)₄, dioxane, H₂O, 130 ºC microwave; (c) TFA, CH₂Cl₂; (d) HCl, Et₂O, 12% over 4 steps.

Synthesis of the pyridylisoxazole 9 is shown in Scheme 6. Treatment of 5-bromo-2-hydroxyxycotinic acid (35) with thionyl chloride and methanol with a catalytic amount of DMF provided the corresponding methyl ester. Formation of the N-hydroxyamide followed by sodium hydroxide-promoted cyclization gave the fused isoxazolopyridinone 36. Successive Mitsunobu reaction with alcohol 18, Suzuki coupling, deprotection and HCl salt formation gave the target 9.

Scheme 6. Reagents and conditions: (a) SOCl₂, DMF, MeOH, 65 ºC, 98%; (b) NH₂OH, dioxane, aq. NaOH, 94%; (c) NaOH, 70 ºC, 59%; (d) PPh₃, DIAD, THF, rt, 68%; (e) 2-chlorobenzeneboronic acid, Cs₂, Pd(PPh₃)₄, DMF, H₂O, 100 ºC; (f) TFA, CH₂Cl₂; (g) HCl, Et₂O, 18% over 3 steps.

Scheme 7 shows the synthesis of inhibitor 10. Conversion of 38 to the corresponding methyl ester followed by bromination gave compound 39. Suzuki coupling followed by treatment with hydroxylamine under basic conditions gave compound 40. Cyclization with carbonyl-imidazole gave intermediate 41 which was treated with alcohol 18 under Mitsunobu conditions, followed by deprotection and formation of the HCl salt to give 10.

Scheme 7. Reagents and conditions: (a) TMSCHN₂, CH₂Cl₂, MeOH; (b) Br₂, H₂O, 42% over 2 steps; (c) 2-chlorobenzeneboronic acid, Cs₂, Pd(PPh₃)₄, DMF, 100 ºC, 16%; (d) NH₂OH, dioxane, aq. NaOH; (e) CDT, Et₂N, CH₂Cl₂, 23% over 2 steps; (f) PPh₃, DIAD, THF, rt; (g) TFA, CH₂Cl₂; (h) HCl, Et₂O, 29% over 3 steps.

The cyclized analogs of compound 1 were initially tested in a DNA gyrase biochemical assay and profiled against a panel of Gram-negative organisms, in addition to a mammalian cytotoxicity assay in mouse L5178Y TK +/− cells (Table 1). Inhibitor 2, representing ring closure strategy a (Figure 1), retained modest inhibition of DNA gyrase and demonstrated an improvement in cytotoxicity over the parent thiophene analog. Unfortunately, no whole cell antibiotic activity was observed across the range of wild-type (WT) Gram-negative pathogens tested (MICs ≥128 µg/mL). Activity could be detected when efflux was impaired by deletion of tolC in Escherichia coli (E. coli αtolIC MIC = 2-4 µg/mL) suggesting that efflux was a contributor to the lack of WT antibacterial activity. On-target activity at DNA gyrase was confirmed by an 8-fold shift in MIC against an E. coli αtolIC GyrB E793K mutant target (a mode-of-action tool strain) relative to the isogenic parental strain. Due to the lack of activity against WT strains, additional analogs in this series were not pursued.

Initial investigation of the scope of ring closure strategy b (Figure 1) resulted in compounds 3-8, representing 3-O-alkylated benzoisoxazoles, 3-O- and 3-N-alkylated indazoles, and 1-N-alkylated isoquiones. The benzoisoxazole 3 demonstrated promising DNA gyrase biochemical activity and modest (8-16 µg/mL) activity against WT E. coli, Acinetobacter baumannii (A. baumannii), with activity against Klebsiella pneumoniae (K. pneumoniae) and Pseudomonas aeruginosa (P. aeruginosa) being considerably weaker (MICs ≥128 µg/mL, not shown). Very potent activity was observed in the efflux knock-out stains and the ≥16-fold shift in MIC in the GyrB E793K mutant demonstrated on-target activity (Table 1).

To confirm that the binding mode of compound 3, we initially solved a 2.7Å co-crystal structure with S. aureus DNA gyrase and 20-12p-8 double nicked DNA. This showed that compound 3 indeed bound as expected (Figure 3B) to the same allosteric site as compound 1. The DNA used for this structure contained artificial nicks at each binding site, and the central four base-pairs of the DNA were not well defined in electron density maps. To try to obtain a clear view of a DNA-cleavage complex with compound 3, we showed that compound 3 bound as seen in the 2.7Å structure, as expected (data not shown). We have previously reported that compound 1 gave both single- and double-stranded DNA cleavage with E. coli DNA gyrase. Using a fusion truncate of DNA gyrase from K. pneumoniae, we showed that compound 3 also enhanced single-stranded and double-stranded DNA cleavage products (data not shown) indicating the mechanism of action of compound 3 is via stabilization of ternary cleaved complexes. In addition to
inhibition of DNA gyrase (IC\textsubscript{50} = 0.16 µM, Table 1), compound 3 weakly inhibited \textit{E. coli} TopoIV activity (IC\textsubscript{50} was ~ 90 µM in a decatenation of kinetoplast DNA inhibition assay\textsuperscript{16}) and induced TopoIV-mediated DNA cleavage breaks (data not shown), and was also weakly active against the human enzyme (IC\textsubscript{50} was ~210 µM against human topoisomerase II\textalpha in a decatenation of kDNA inhibition assay\textsuperscript{16}). Encouraged by the initial data of compound 3 and validation of the ring closure b hypothesis, several related 5,5 and 5,6 fused heterocycles were prepared (4-8). Relative to compound 3, these analogs had reduced DNA gyrase activity and weaker antimicrobial activity in WT and efflux knock-out strains of \textit{E. coli} and WT \textit{A. baumannii} (Table 1). Interestingly, slight improvements in activity against \textit{K. pneumoniae} relative to compound 3 were observed for some analogs. Like compound 3, none of the newer analogs possessed activity against WT \textit{P. aeruginosa} (MIC >128 µg/mL). Unfortunately, whole cell on-target activity was not demonstrated for these analogs as no MIC shift was observed in the \textit{E. coli} GyrB E793K mutant, perhaps suggesting that some of the activity observed is not solely due to inhibition of DNA gyrase.

As analogs 4-98 displayed disappointing antibacterial activities, we returned to the benzisoxazole scaffold of compound 3 and synthesized the three aza-analogs 9-11 and the guanidine 12. Although all analogs retained modest to good levels of activity against DNA gyrase, none showed an improvement in antibacterial activity in either WT or efflux strains of \textit{E. coli} (12 gave a 2-fold improvement against \textit{A. baumannii}, Table 2). No activity (MIC >128 µg/mL) was observed against \textit{K. pneumoniae} and \textit{P. aeruginosa}, except for compound 12 which gave an MIC of 16 µg/mL against both \textit{K. pneumoniae} and \textit{P. aeruginosa} (data not shown). On-target activity was maintained for some (e.g. 9 and 11) but was less clear for analogs 10 and 12. Indeed, the broader spectrum activity of compound 12 could be due to an additional non-specific mechanism of action, potentially related to the guanidine substituent which is installed in an effort to alter the polarity of the series.

Despite the disappointing antibacterial activity, we tested several analogs against cardiac ion channels to determine if rigidifying the core would impact these activities (Table 2). Although 3 did have attenuated inhibition of both the hERG\textsuperscript{17} and Na\textsubscript{v}1.5\textsuperscript{18} channels compared to compound 1 (Table 2), the effect was not dramatic. Compounds 9, 10, and 12 also retained relatively potent inhibition against one or both of the cardiac ion channels tested, highlighting the difficulty in optimizing safety parameters in addition to activity in antibacterial research and development.

In summary, we have synthesized and tested several 5,6 and 6,6 fused core analogs of thiophene antibacterial 1. Although both initial design hypotheses were successful in delivering analogs with \textit{in vitro} activity against DNA gyrase, none of the analogs had improved whole cell antibacterial activity relative to the parent compound 1. Moreover, several liabilities of the series, specifically cytotoxicity and cardiac ion channel activity, were not mitigated with this ring-fusion strategy. As such, we stopped work on the present ring-fusion approach. A complete account of our efforts on monocyclic series, including liabilities and reasons for termination, will be reported separately.

\textbf{Figure 3.} A) 2.22 Å crystal structure (pdb code: 5NPP) of 1 (yellow carbons) in complex with \textit{S. aureus} DNA gyrase (GyrA blue carbons, GyrB magenta carbons) and DNA (green atom, lower left)\textsuperscript{7}. Nitrogens blue, oxygens red, phosphorous orange chlorine green. Semi-transparent surface is shown on GyrA but not GyrB. Semi-transparent surface is shown on GyrA but not GyrB. Hydrogen bonds (dotted yellow lines) from E634 (=E793 in \textit{E. coli}) go to 1 and R630. R630 also forms H-bonds to 1. B) 2.7 Å crystal structure of 3 (white carbons) in complex with \textit{S. aureus} DNA gyrase and DNA (pdb code: 6QX1). Hydrogen bond is shown as black dotted line. Note, the hydrogen bonds from R630 to 3 is similar to that in panel A from R630 to 1; but no H-bonds are observed to E634 with 3 (maybe because of a small movement of the end of the side-chain of R630). C) Superposition of crystal structures in A and B. Figure drawn with pymol (The PyMOL Molecular Graphics System, Schrödinger, LLC).
Table 1. Biochemical and bacterial susceptibility data for bicyclic gyrase inhibitors 1-8.

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<th>E.coli 7623 ΔtolC GyrB E793K IC₅₀ (µg/mL)</th>
<th>K. pneumoniae 1161486 IC₅₀ (µM)</th>
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Table 2. Biochemical, bacterial susceptibility and cardiac ion channel data for 5,6-fused gyrase inhibitors 3 and 9-12.

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References and notes

6. See: http://nd4bb-enable.eu/


Engberg, Y. High-throughput DNA gyrase biochemical assay was developed based on a preferential enhancement of fluorescence of a H19 dye following binding to supercoiled DNA compared to relaxed DNA. Test compounds were incubated at room temperature for 1 hour with 15 μg/ml relaxed pBR322 DNA (Topoquin or Inspiralis), 60 mM of His-tagged E.coli DNA gyrase and 2.5 mM ATP in an assay buffer of 20 mM Tris-HCl, 35 mM Na2L6Ac (pH 8), 8 mM MgCl2, 4.6% glycerol, 0.005% Brij35 and 1 mM DTT. Relaxed and supercoiled DNA forms were detected following incubation with a 1:1 in a 400 dilution of H19 dye (ProFoldin) and fluorescence intensity reading with Ex: 485 nm and Em: 530 nm. Data was analyzed using Sophion Qpatch software version 5.0. Data was collated using Microsoft Excel and all subsequent analysis was performed using SignalPlot Version 13.0 (2015). (Enzymes and substrates provided by Inspiralis Biologics). 


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analyses were done using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, California USA, www.graphpad.com). Measurements were fit to a four-parameter logistic fit for dose-response curves: 

\[
y = \text{bottom} + \frac{\text{top} - \text{bottom}}{1 + 10^{((\log EC_{50} - x) \times \text{Hill slope})}}
\]