The Naphthoquinone Diospyrin Is an Inhibitor of DNA Gyrase with a Novel Mechanism of Action*⑤

Shantanu Karkare[‡1], **Terence T. H. Chung**[‡], **Frederic Collin**[‡], **Lesley A. Mitchenall**[‡], **Adam R. McKay**[§], **Sandra J. Greive**[‡2], **Jacobus J. M. Meyer**[¶], **Namrita Lall**[¶], and **Anthony Maxwell**[‡3]

From the [‡]*Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, United Kingdom, the* [§]*Department of Chemistry, University College London, London WC1H 0AJ, United Kingdom, and the* [¶]*Department of Plant Science, University of Pretoria, Pretoria 0002, South Africa*

Background: New antibacterial compounds are urgently needed; DNA gyrase is a well-validated target.
Results: Diospyrin and other naphthoquinones inhibit DNA gyrase by binding to a novel site in the B subunit.
Conclusion: Naphthoquinones are inhibitors of gyrase with a novel mechanism of action.
Significance: Naphthoquinones have potential as antibacterial compounds against TB.

Tuberculosis and other bacterial diseases represent a significant threat to human health. The DNA topoisomerases are excellent targets for chemotherapy, and DNA gyrase in particular is a well-validated target for antibacterial agents. Naphthoquinones (*e.g.* diospyrin and 7-methyljuglone) have been shown to have therapeutic potential, particularly against *Mycobacterium tuberculosis*. We have found that these compounds are inhibitors of the supercoiling reaction catalyzed by *M. tuberculosis* gyrase and other gyrases. Our evidence strongly suggests that the compounds bind to the N-terminal domain of GyrB, which contains the ATPase active site, but are not competitive inhibitors of the ATPase reaction. We propose that naphthoquinones bind to GyrB at a novel site close to the ATPase site. This novel mode of action could be exploited to develop new antibacterial agents.

Infectious diseases caused by bacterial pathogens are a serious and growing problem. For example, tuberculosis (TB)[4] is the most deadly infectious disease in the world with ~2 billion people infected and >1 million deaths every year (1). Current treatments involve therapy over a long period (~6 months), and there are serious problems with drug-resistant strains (multidrug-resistant TB and extensively drug resistant-TB). One strategy for developing new antibacterials is to seek new targets. However, despite more than a decade of research driven by genomics and proteomics, and enormous expenditure, very few promising new targets have emerged. It appears that Nature, through millions of years of evolutionary warfare, has already identified the "good bacterial targets," which include the ribosome, cell wall synthesis enzymes, and DNA gyrase (2).

DNA gyrase is a DNA topoisomerase that is present in bacteria and plants, but not animals, and has been widely exploited as a target for antimicrobial chemotherapy (3, 4). DNA topoisomerases are enzymes that catalyze changes in the topology of DNA and are essential to all cells (5). They are classified into two types, depending upon whether their reactions involve transient single- (type I) or double- (type II) strand breaks in DNA. All topoisomerases can relax supercoiled DNA but gyrase, a type II enzyme, can also introduce negative supercoils in a reaction coupled to ATP hydrolysis (4, 5). Gyrase consists of two subunits, GyrA and GyrB, which form an A_2B_2 complex in the active enzyme in which a segment of DNA is wrapped around the protein. The GyrA subunit interacts with DNA and contains the active-site tyrosine responsible for DNA cleavage and the formation of a protein-DNA covalent bond during the reaction cycle. GyrB also interacts with DNA and contains the ATPase active site. Gyrase shares a number of features with other type II topoisomerases but is distinct in its ability to wrap DNA and harness the free energy of ATP hydrolysis to introduce negative supercoils into DNA. The uniqueness of gyrase has made it a successful target for antibacterial agents. Fluoroquinolones (*e.g.* ciprofloxacin, moxifloxacin) target gyrase and are highly successful clinical agents that have been used against tuberculosis. However, despite their efficacy, fluoroquinolone-resistant TB is a serious problem (6).

Most of our current information about DNA gyrase concerns the *Escherichia coli* enzyme, but it is clear that enzymes from other bacteria have important differences that need to be investigated to exploit their potential as drug targets for specific diseases. DNA gyrase from *Mycobacterium tuberculosis* has a number of distinct features that warrant investigation in their own right and that may be exploitable for the targeting of this enzyme (7, 8). For example, *M. tuberculosis* gyrase has been found to be a potent decatenase, in contrast to most other gyrases (8–10), reflecting the fact that *M. tuberculosis* lacks

⑤ This article contains supplemental Figs. S1–S5, Table S1, and additional references.
[1] Present address: Novartis Institute of Biomedical Research, Basel 4056, Switzerland.
[2] Present address: Inspiralis Ltd., Norwich Bioincubator, Norwich Research Park, Colney Lane, Norwich NR4 7UH, UK.
[3] To whom correspondence should be addressed. Tel.: 44-1603-450771; Fax: 44-1603-450018, E-mail: tony.maxwell@jic.ac.uk.
[4] The abbreviations used are: TB, tuberculosis; MIC, minimum inhibitory concentration; nano-ESI, nanoflow electrospray ionization; SPR, surface plasmon resonance; topo, topoisomerase.
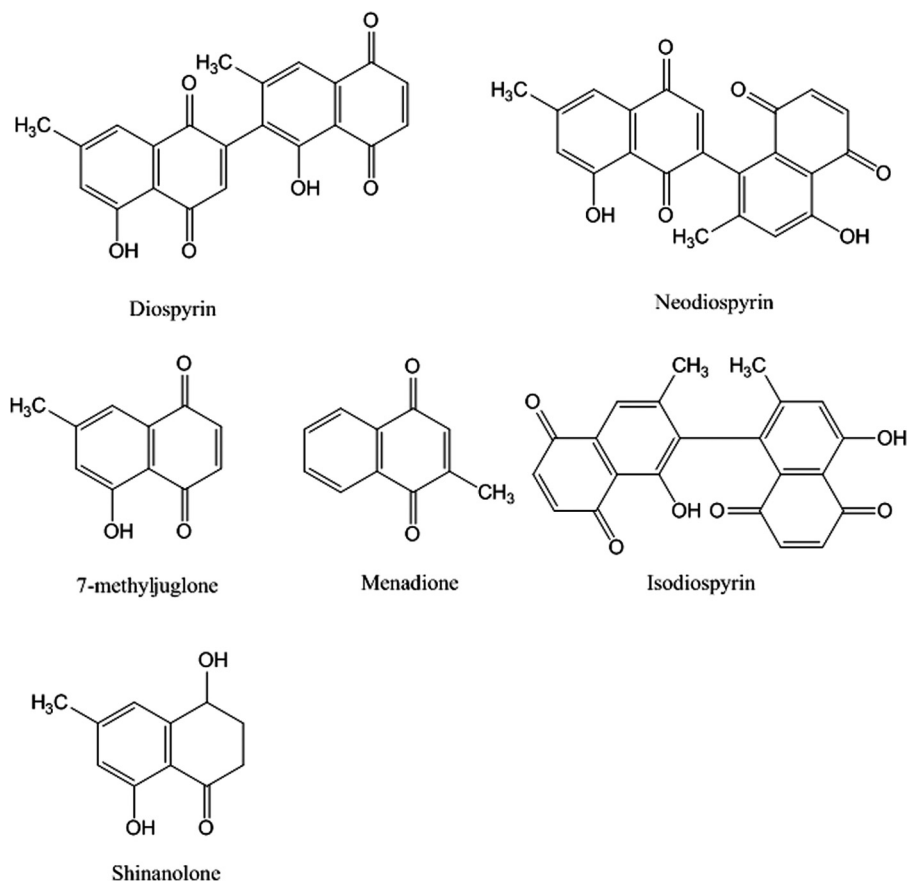
FIGURE 1. **Structures of naphthoquinones.**

topo IV, which is the predominant decatenating enzyme in most bacteria (11). Recent advances in *M. tuberculosis* gyrase have included the structures of the N- and C-terminal domains of GyrA and the C-terminal domain of GyrB (12–14), the identification of DNA-binding residues in the C-terminal domain of GyrA (15), and the development of monoclonal antibodies that specifically target the enzyme as potential therapeutic agents (16). Recently a potential Ca^{2+}-binding site has been identified in *M. tuberculosis* GyrA, which may have a regulatory role (17).

Extracts from plants used in traditional medicine provide a source for novel compounds that may have antibacterial properties. Lall and Meyer have analyzed the antibacterial properties of the South African tree *Euclea natalensis*. This plant, also known as the "toothbrush tree" (native South Africans use its twigs as toothbrushes), has been used extensively to treat a variety of medical complaints (*e.g.* bronchitis, pleurisy, venereal disease) and in oral health (18). Lall and Meyer showed that crude extracts from *E. natalensis* were active against drug-sensitive and drug-resistance strains of *M. tuberculosis* (19). They later reported that the active component in these extracts was the naphthoquinone diospyrin (Fig. 1) (20). Naphthoquinones are widely distributed in nature, and their presence in many plants is the basis for some folk medicines (21). They have been implicated in the treatment of a variety of diseases including urinary tract infections, trypanosome diseases, and tuberculosis (21, 22). Diospyrin, a bisnaphthoquinone (Fig. 1), has previously been found to be an inhibitor of DNA topoisomerase (topo) I from *Leishmania donovani* and can stabilize the topo I-DNA

cleavage complex (23); isodiospyrin was found to be an inhibitor of human topo I, but did not stabilize the cleavage complex (24); 7-methlyjuglone was shown to be a subversive substrate for *M. tuberculosis* mycothiol disulfide reductase (25). In addition, there are a number of reports of quinolones interacting with eukaryotic type II topoisomerases (*e.g.* human topo IIα). For example, several naphthoquinones, including juglone, have been shown to inhibit topo II and stabilize the cleavage complex (26); these compounds react with thiol groups on the protein.

The lack of a clear target definition and the observation of the efficacy of diospyrin against drug-sensitive and drug-resistance strains of *M. tuberculosis* (19), prompted us to test this and other naphthoquinones against *M. tuberculosis* DNA gyrase. We have found that these compounds can inhibit gyrase and that they target the enzyme by a novel mechanism, raising the possibility of developing these compounds as potential anti-TB agents.

EXPERIMENTAL PROCEDURES

Enzymes and DNA—*M. tuberculosis* and *E. coli* gyrases and the N-terminal domain of *E. coli* GyrB (GyrB43) were prepared as described previously (17, 27, 28). *Staphylococcus aureus* WCUH29 *gyrA*, *gyrB*, *parC*, and *parE* genes in pET vectors were gifts from Hiroshi Hiasa (University of Minnesota). They were re-cloned into plasmid pET11 (Novagen) and the proteins expressed in *E. coli* Rosetta 2 (DE3) pLysS (Novagen). Both proteins were purified using an Äkta system (GE Healthcare) using

Q-Sepharose, heparin-Sepharose, and phenyl-Sepharose columns to >95% purity.

Enzyme Assays—*M. tuberculosis* gyrase supercoiling, relaxation, and decatenation assays were carried out as described previously (17); cleavage assays were carried out as per relaxation assays ($\pm$ATP) except that following incubation at 37 °C, SDS and proteinase K were added (to 0.2% and 0.1 mg/ml, respectively) and the incubation was continued at 37 °C for 30 min before loading onto an agarose gel for analysis. *E. coli* gyrase supercoiling assays were performed as described (29). *S. aureus* gyrase and topo IV assays were carried out as described (30), apart from the buffer exchange step prior to electrophoresis, which was omitted; samples were loaded onto agarose gels and left for 30 min prior to electrophoresis to allow diffusion of salt. ATPase assays were performed using a linked assay as described (27), except that assays were carried out in microtiter plates, and A_{340} values were measured continuously using an absorbance plate reader. Limited proteolysis experiments were performed as described previously (17).

Mass Spectrometry and SPR—Nanoflow electrospray ionization mass spectrometry (nano-ESI MS) and surface plasmon resonance (SPR) experiments were performed as described previously (31, 32), with the exception that SPR data collection used a Biacore T-100 instrument (GE Healthcare), and regeneration was carried out with 4.5 mM sodium hydroxide. Protein and drug stock solutions were filtered through 0.1-μm spin filters (Merck Millipore) prior to injection over the chip surface to prevent damage to the microfluidics caused by insoluble aggregates. Data were analyzed was using the BIAEvaluation software and displayed using Sigma Plot (Systat Software Inc.).

RESULTS

Inhibition of DNA Gyrase by Naphthoquinones—To determine whether diospyrin and 7-methyljugolone might target *M. tuberculosis* DNA gyrase, we assessed their effect on the gyrase supercoiling reaction (Fig. 2). Both compounds inhibit this reaction with IC$_{50}$ values of ~15 μM (diospyrin) and ~30 μM (7-methyljugolone); other naphthoquinones were also tested in this reaction (Table 1; the IC$_{50}$ values of novobiocin and ciprofloxacin against *M. tuberculosis* gyrase are given for comparison). We tested diospyrin against *M. tuberculosis* gyrase in relaxation reactions and found that this reaction is also inhibited by this compound with an IC$_{50}$ similar to that for supercoiling (supplemental Fig. S1). We also tested diospyrin and 7-methyljuglone for their ability to inhibit DNA supercoiling by *E. coli* and *S. aureus* gyrases. We found that both of these enzymes were inhibited by these compounds with approximate IC$_{50}$ values as follows: *E. coli*, 4 μM (diospyrin) and 30 μM (7-methyljuglone); *S. aureus*, 8 μM (diospyrin) and 60 μM (7-methyljuglone) (Table 2 and supplemental Fig. S2). As our assays contained 4 mM DTT (*i.e.* reducing conditions), and it has been reported that quinone inhibitors of topo II are less active under reducing conditions (33), we attempted to carry out experiments without reducing agents. However, we found that *M. tuberculosis* gyrase was inactive in the absence of reducing agents and even with 50 μM DTT showed no activity (data not shown). *M. tuberculosis* gyrase supercoiling assays carried out with diospyrin and 7-methyljugone in the presence of 2 mM
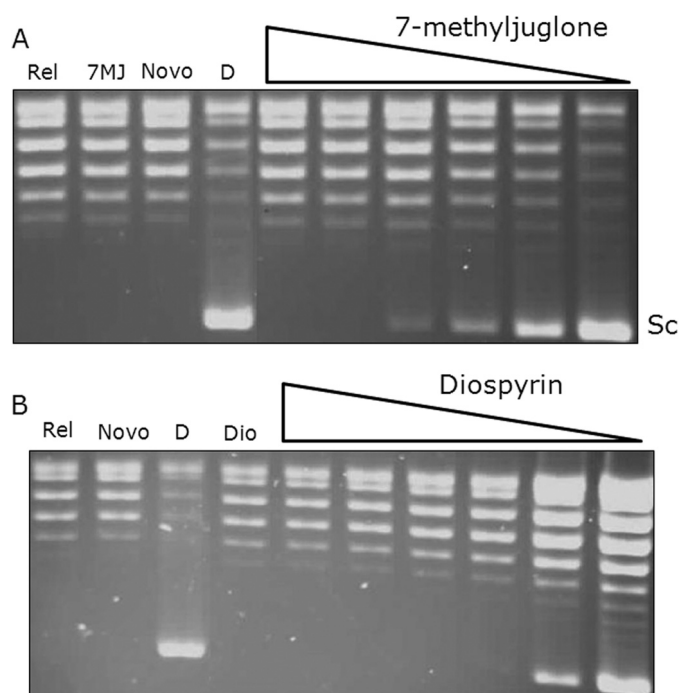

FIGURE 2. **Inhibition of *M. tuberculosis* gyrase supercoiling by 7-methyljuglone and diospyrin.** *A*, inhibition of supercoiling in the presence of 7-methyljuglone (1, 10, 30, 50, 100, 200 μM); *Rel* is relaxed DNA; *7MJ* is 7-methyljuglone (no enzyme); *Novo* is gyrase in the presence of 5 μM novobiocin; *D* is gyrase in the presence of 3% dimethyl sulfoxide; *Sc* indicates the position of the supercoiled DNA band. All 7-methyljuglone samples also contained 3% dimethyl sulfoxide. *B*, inhibition of supercoiling in the presence of diospyrin (concentrations as above).

TABLE 1

IC$_{50}$ values for the inhibition of supercoiling by *M. tuberculosis* gyrase by naphthoquinones

Compound	IC$_{50}$
	μM
Diospyrin	15
7-Methyljuglone	30
Neodiospyrin	50
Isodiospyrin	100
Menadione	>200
Shinanolone	>200
Ciprofloxacin	10
Novobiocin	1

TABLE 2

Inhibition of DNA gyrases from *S. aureus*, *E. coli*, and *M. tuberculosis* by diospyrin and 7-methyljuglone

Compound	IC$_{50}$ for gyrase supercoiling inhibition		
	S. aureus	*E. coli*	*M. tuberculosis*
	μM	μM	μM
Diospyrin	8	4	15
7-Methyljuglone	60	30	30

β-mercaptoethanol gave results similar to those carried out with 4 mM DTT. In contrast we have found that *S. aureus* gyrase is active in the presence of a low concentration of DTT (50 μM), and we found inhibition by diospyrin and 7-methyljuglone similar to that we had found at 4 mM DTT (data not shown). Taken together, it seems that there is no significant effect of reducing agents on the inhibitory effects of diospyrin and 7-methyljuglone for gyrase.

DNA topoisomerase IV (topo IV) is also the target of a number of antibacterial agents (34, 35), however *M. tuberculosis*
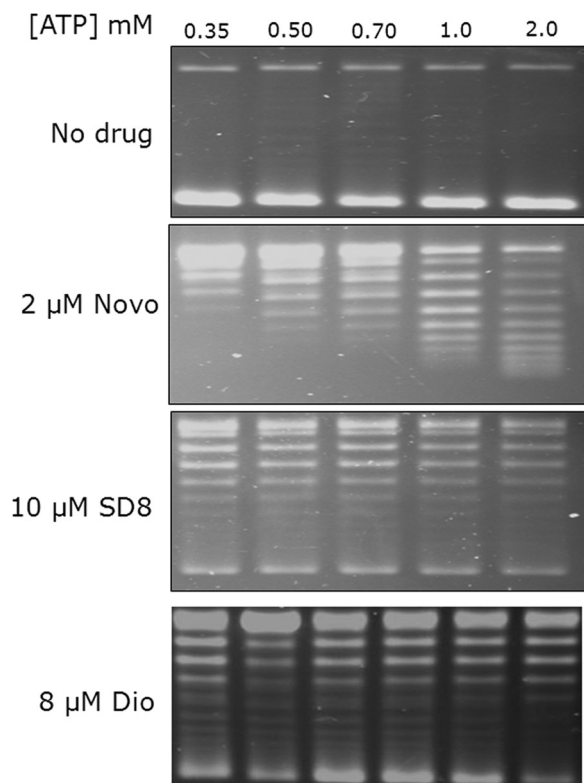
FIGURE 3. **Effect of ATP on the inhibition of *M. tuberculosis* gyrase by novobiocin, simocyclinone, and diospyrin.** The indicated concentrations of ATP were added to *M. tuberculosis* gyrase supercoiling reactions containing the indicated amounts of novobiocin (*Novo*), simocyclinone D8 (*SD8*), and diospyrin (*Dio*).

FIGURE 4. **Inhibition of *S. aureus* GyrB ATPase by novobiocin and diospyrin.** The rates of ATP hydrolysis (s^{-1}) by GyrB (0.6 μM) in the presence of novobiocin (*squares*) and diospyrin (*circles*) were determined. Data were fitted using SigmaPlot 12.3 to schemes involving the binding of two ligands to inhibit ATPase activity (novobiocin) or a single ligand (diospyrin).

lacks this enzyme (36); *M. tuberculosis* gyrase has evolved to carry out activities catalyzed by gyrase and topo IV in other bacteria. We tested naphthoquinones against *S. aureus* topo IV and found that in general naphthoquinones favored gyrase over topo IV, for example the IC_{50} for diospyrin is 4–8 times higher for *S. aureus* topo IV than for gyrase (supplemental Table S1).

The success of fluoroquinolones, such as ciprofloxacin, as antibacterial agents is due to the fact that they stabilize the cleavage complex between gyrase and DNA (37–39), in which the GyrA protein is covalently attached to the 5′-end of the cleaved DNA. We tested diospyrin and 7-methyljuglone in cleavage-complex assays under conditions (±ATP) where ciprofloxacin stabilizes the cleavage complex between *M. tuberculosis* gyrase and DNA. We found no evidence of stabilization of the gyrase-DNA cleavage complex by these agents (data not shown); this is in contrast with results obtained with naphthoquinones (*e.g.* juglone) and topo II (26).

The fact that diospyrin inhibits ATP-independent relaxation by gyrase suggests that naphthoquinones are unlikely to be ATPase inhibitors. We tested diospyrin in gyrase supercoiling reactions at a range of ATP concentrations (Fig. 3). (This approach has been used previously to show that simocyclinones are not inhibitors of the ATPase reaction of gyrase (32).) We found that whereas inhibition by novobiocin (a well-established inhibitor of the gyrase ATPase reaction) could be abrogated by ATP, inhibition by diospyrin and simocyclinone could not, supporting the contention that diospyrin is not a competitive inhibitor of the gyrase ATPase reaction. We directly tested the
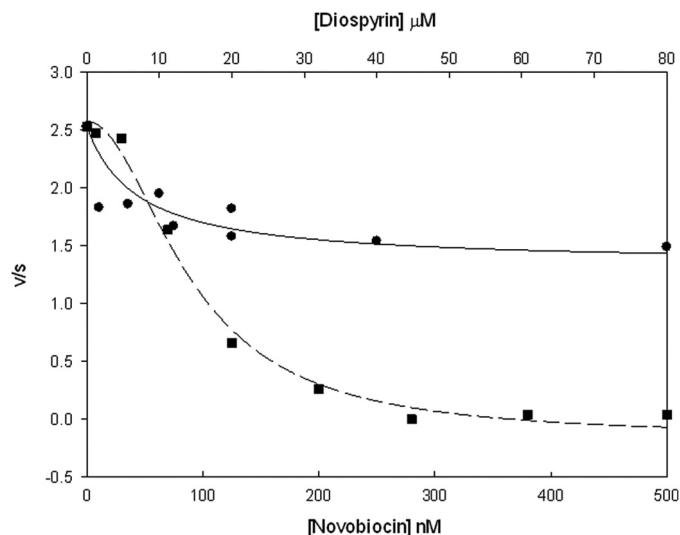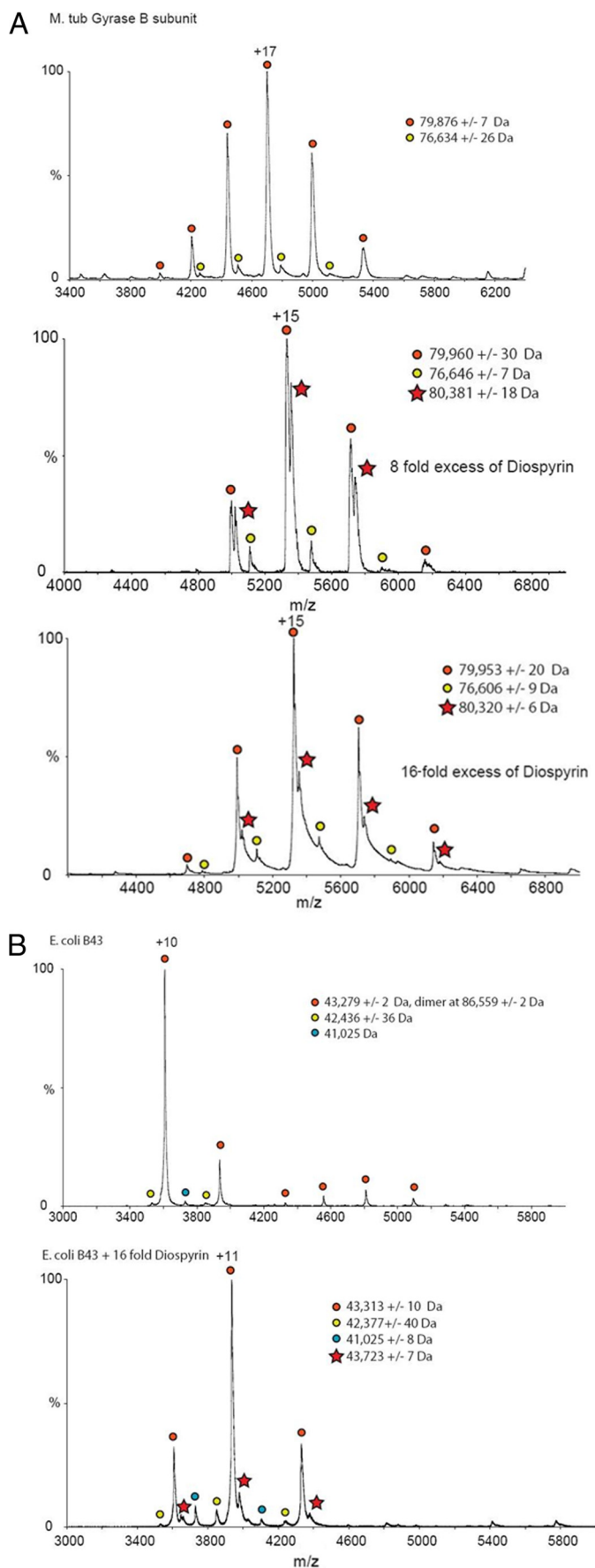
effect of diospyrin on the ATPase reaction of *M. tuberculosis* GyrB, but we found that the intrinsic ATPase activity of this protein to be very low, making this experiment problematic (data not shown). However, we found that the ATPase activity of *S. aureus* GyrB could be measured more readily and showed that, whereas this reaction is completely inhibited by novobiocin, diospyrin could only inhibit the reaction by ~50% (Fig. 4); similar results were obtained with *S. aureus* GyrB and 7-methyljuglone (data not shown). For novobiocin, the dependence of rate on inhibitor concentration was sigmoidal, so the data were fitted to a scheme involving the binding of two ligands to the enzyme to achieve inhibition; in the case of diospyrin the data showed a hyperbolic dependence on inhibitor concentration consistent with the binding of one ligand molecule/enzyme to achieve inhibition (Fig. 4). These data suggest that diospyrin is not a competitive inhibitor of the ATPase reaction of GyrB, but that its site of action is likely to be within the GyrB N-terminal domain and can allosterically affect the ATPase reaction (see below).

The Binding Site of Diospyrin Is in GyrB—The biochemical data presented above suggest that diospyrin can inhibit the topoisomerase reaction of gyrase and that it may act by binding to the GyrB subunit. We used three further approaches to explore this. In previous work we have used limited tryptic digestion to probe ligand-binding sites in gyrase (17, 40–42). Limited digestion of *M. tuberculosis* GyrA with trypsin revealed little or no difference with and without diospyrin (supplemental Fig. S3*A*), whereas differences were observed in the case of GyrB (supplemental Fig. S3*B*). In the case of *E. coli* GyrB43 (the N-terminal domain of GyrB), significant differences in the digestion pattern were also observed with and without diospyrin (supplemental Fig. S3*C*). These data support the idea that diospyrin may bind to the N-terminal domain of GyrB, which houses the ATPase active site (27, 44).

Nano-ESI MS has been used successfully to analyze the noncovalent interaction between proteins and ligands (45).

FIGURE 5. **Mass spectrometry analysis of the binding of diospyrin to GyrB.**
A: *top*, *M. tuberculosis* GyrB (*red* and *yellow circles* indicate two different
charged species); *middle*, *M. tuberculosis* GyrB with 8-fold excess diospyrin
(*red stars* indicate species containing diospyrin); *bottom*, *M. tuberculosis* GyrB
with 16-fold excess diospyrin. *B*: *top*, *E. coli* GyrB43 (N-terminal domain; *red*,
yellow, and *blue circles* indicate three different charged species); *bottom*,
E. coli GyrB43 with 16-fold excess diospyrin (*red stars* indicate species contain-
ing diospyrin).

Recently, we have used this method to characterize the interac-
tion between the N-terminal domain of *E. coli* GyrA and the
antibiotic simocyclinone D8 (29, 31). We have now analyzed
the interaction between diospyrin and gyrase by this method
(Fig. 5; note that all nano-ESI MS experiments were performed
in the absence of DTT). We found no evidence for interaction
between diospyrin and *M. tuberculosis* GyrA (data not shown),
but spectra of samples with *M. tuberculosis* GyrB and diospyrin
showed additional peaks consistent with a complex between
the two (Fig. 5*A*). In each spectrum two sets of charged species
are apparent (indicated by *red* and *yellow circles*), which corre-
spond to the GyrB monomer: observed molecular mass =
$79,876 \pm 7$ Da (calculated = 79,263) and a lower molecular
mass contaminant, which is likely to be a truncated form of
GyrB. In the spectra in the presence of diospyrin, additional
species are present (*red stars*), which correspond to the GyrB
monomer with bound ligand: observed molecular masses =
$80,381 \pm 18$ Da and $80,320 \pm 6$ Da (the molecular mass of
diospyrin is 374 Da). *E. coli* GyrB43 shows three different
charged species the largest of which corresponds to the GyrB43
monomer (observed molecular mass = $43,272 \pm 2$ Da (calcu-
lated = 43,024); the other two minor species are likely to cor-
respond to protein fragments or contaminants. In the presence
of diospyrin a further species (MW $43,313 \pm 10$ Da) appears
corresponding to the GyrB43 monomer with bound diospyrin
(Fig. 5*B*). No evidence for interaction between diospyrin and
the C-terminal domain of *E. coli* GyrB (GyrB47) was found
(supplemental Fig. S4). Taken together, it seems very likely that
the binding site for diospyrin lies within the N-terminal domain
of GyrB.

To further evaluate the GyrB-diospyrin interaction, SPR was
employed. Control experiments showed that novobiocin bound
to *E. coli* GyrB43 and *M. tuberculosis* GyrB with equilibrium
binding constant (K_D) values of 10^{-8} to 10^{-7} M (supplemental
Fig. S5), consistent with earlier SPR studies on the interaction of
this drug with *E. coli* GyrB (46). In experiments with diospyrin,
we observed a specific interaction with *E. coli* GyrB43 (Fig. 6),
with an upper limit for the K_D determined to be 10^{-4} M. Further
refinement of this value was impractical due to the tendency of
diospyrin to aggregate on the *E. coli* GyrB chip surface at high
concentrations (>50 μM; supplemental Fig. S5).

From these data it seems very likely that the binding site for
diospyrin lies in the N-terminal domain of GyrB, but does not
overlap the ATP-binding site. This suggests a mode of interac-
tion that is distinct from that of other known gyrase inhibitors.

DISCUSSION

There is a significant and immediate threat from infections
due to pathogenic bacteria that is exacerbated by the prevalence
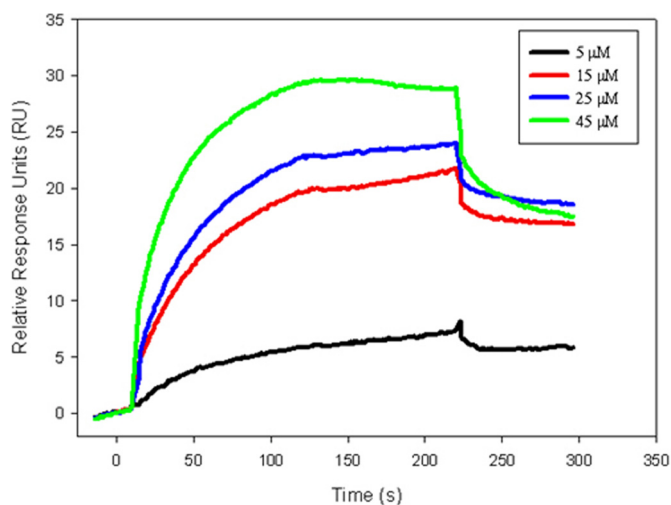of antibiotic-resistant bacteria and diminution in the drug-dis-

FIGURE 6. **SPR analysis of the binding of diospyrin to *E. coli* GyrB43.** Sensograms showing the interaction of diospyrin at selected concentrations (5 (*black*), 15 (*red*), 25 (*blue*), and 45 (*green*) μM) with *E. coli* GyrB43.

covery efforts of large pharmaceutical companies (47, 48). This means that there is an urgent need for new compounds with antibacterial potential. Plants are a rich and largely untapped source of antibacterials (49–51), although plant extracts have been used medicinally for centuries. The toothbrush tree (*E. natalensis*), for example, has been traditionally utilized for its medicinal properties by native South Africans: the roots are chewed for oral hygiene and for the treatment of bronchitis, pleurisy, and chronic asthma (18). Extracts from this plant have been found to show antibacterial activities, particularly against Gram-positive organisms (18), including *M. tuberculosis* (19). Diospyrin isolated from *E. natalensis* was shown to have activity against drug-sensitive and drug-resistant *M. tuberculosis* strains (20). Other naphthoquinones were also tested against *M. tuberculosis* strains and showed the following MIC values (in order of potency): 7-methyljuglone (0.5 μg/ml; 2.6 μM), diospyrin (8 μg/ml; 21.4 μM), isodiospyrin (10 μg/ml; 26.7 μM), and neodiospyrin (10 μg/ml; 26.7 μM). However, the *in vivo* target for these compounds is not clear, with 7-methyljuglone having been identified as a subversive substrate for mycothiol disulfide reductase (25), diospyrin found to be an inhibitor of topo I from *L. donovani* (23), and isodiospyrin found to be an inhibitor of human topo I (24). In an attempt to address this issue, we have tested diospyrin, 7-methyljugulone, and other naphthoquinones against gyrases from *M. tuberculosis, S. aureus*, and *E. coli.*

We have found that diospyrin and other naphthoquinones are inhibitors of the DNA supercoiling reaction of DNA gyrase with IC_{50} values that are comparable with the reported MICs. For example, we found the IC_{50} value for diospyrin against *M. tuberculosis* gyrase to be ~15 μM, which compares with an MIC of ~20 μM (8 μg/ml). Interestingly diospyrin also inhibits the DNA relaxation reaction of gyrase despite our evidence suggesting binding to the ATPase domain of GyrB, which is not required for relaxation activity. Our ATPase experiments suggest that diospyrin is an allosteric inhibitor of this reaction, and we propose that it binds to the N-terminal domain of GyrB close to but not overlapping with the ATPase active site. Its mode of action may involve stabilization of a conformation of

the N-terminal domain of GyrB that prevents strand passage and thus inhibits both supercoiling and relaxation by gyrase. This mode of action is similar to that proposed by Bender *et al.* (52) for the action of the quinone metabolites of polychlorinated biphenyls on human topo IIα. In the case of the action of naphthoquinones on gyrase, the mechanism would need to involve binding of the inhibitor to the GyrB-N-terminal domain in a manner that would prevent strand passage in either direction and not just prevent clamp closure.

The work in this paper provides evidence that gyrase is a target for naphthoquinones. However, we have not proven that it is the primary *in vivo* target in the antibacterial action of these compounds. One way to show this would be to isolate target-based naphthoquinone-resistant mutations and to establish a correlation between potency and target mutations. Our efforts to isolate such mutations in *E. coli* and *Mycobacterium smegmatis* have so far been unsuccessful. Most gyrase inhibitors found so far either stabilize the DNA cleavage complex by binding to a pocket comprising residues from GyrA, GyrB, and DNA (such as quinolones), or bind to the ATPase site (such as aminocoumarins) (3). Diospyrin does not appear to emulate either of these modes of action but would appear to bind to a novel binding pocket in the N-terminal domain of GyrB, *i.e.* it has a completely new mode of action.

Given that naphthoquinones and other quinone compounds have been found to inhibit eukaryotic topo II, it is interesting to compare their modes of action with that of diospyrin on gyrase. Several naphthoquinones, including juglone, have been shown to inhibit topo II and stabilize the cleavage complex (26); these compounds react with thiol groups on the protein. Lindsey *et al.* (33) found that 1,4-benzoquinone is a strong cleavage-complex stimulator and that the presence of reducing agents led to a reduction in potency, suggesting that that the oxidized form of the compound was the active species. Further, benzoquinone was found to form adducts with Cys residues within the ATPase domain of topo II (Cys-392 and Cys-405 of human topo IIα); mutation of these residues to Ala led to 50% reduction in the potency of the compound (53). More recently, Jacob *et al.* showed that etoposide quinone, a metabolite of etoposide (itself a cleavage-complex inhibitor of topo II (43)), is effective at stabilizing the cleavage complex under nonreducing conditions. Taken together, this work suggests that compounds of this class are cleavage-complex-stabilizing inhibitors of eukaryotic topo II that may act via the formation of adducts with Cys residues in the N terminus (ATPase) domain, although it is unclear as to why mutation of the two key Cys residues in human topo IIα only leads to a ~50% drop in potency.

In comparison, our experiments do not support a similar mode of action. Our results suggest that the naphthoquinone-binding site is within the N-terminal domain of GyrB, but as one of the enzymes examined (*S. aureus* gyrase) lacks Cys residues in the ATPase domain, it is unlikely that covalent adducts with Cys residues form part of the mode of binding. Moreover, in a control experiment, using mass spectrometry, we found no evidence of adduct formation with *M. tuberculosis* gyrase, and, in particular, the Cys residue in the N-terminal domain is unmodified. In addition, we have also found no evidence of

diospyrin and other naphthoquinones stabilizing the gyrase cleavage complex.

A structure of the diospyrin-GyrB complex would clearly be of great value, but, so far, crystallization trials with diospyrin and GyrB and fragments thereof have not yet yielded crystals suitable for diffraction studies. Identification of the naphthoquinone-binding pocket in GyrB would potentiate the development of these compounds as potential anti-TB agents and the design of other molecules that exploit this mode of action.

REFERENCES

1. Young, D. B., Perkins, M. D., Duncan, K., and Barry, C. E., 3rd (2008) Confronting the scientific obstacles to global control of tuberculosis. *J. Clin. Invest.* **118,** 1255–1265
2. Walsh, C. (2000) Molecular mechanisms that confer antibacterial drug resistance. *Nature* **406,** 775–781
3. Collin, F., Karkare, S., and Maxwell, A. (2011) Exploiting bacterial DNA gyrase as a drug target: current state and perspectives. *Appl. Microbiol. Biotechnol.* **92,** 479–497
4. Nöllmann, M., Crisona, N. J., and Arimondo, P. B. (2007) Thirty years of *Escherichia coli* DNA gyrase: from *in vivo* function to single-molecule mechanism. *Biochimie* **89,** 490–499
5. Bates, A. D., and Maxwell, A. (2005) *DNA Topology*, Oxford University Press, Oxford
6. Ginsberg, A. M., and Spigelman, M. (2007) Challenges in tuberculosis drug research and development. *Nat. Med.* **13,** 290–294
7. Mdluli, K., and Ma, Z. (2007) *Mycobacterium tuberculosis* DNA gyrase as a target for drug discovery. *Infect. Disord. Drug Targets* **7,** 159–168
8. Aubry, A., Fisher, L. M., Jarlier, V., and Cambau, E. (2006) First functional characterization of a singly expressed bacterial type II topoisomerase: the enzyme from *Mycobacterium tuberculosis. Biochem. Biophys. Res. Commun.* **348,** 158–165
9. Manjunatha, U. H., Dalal, M., Chatterji, M., Radha, D. R., Visweswariah, S. S., and Nagaraja, V. (2002) Functional characterisation of mycobacterial DNA gyrase: an efficient decatenase. *Nucleic Acids Res.* **30,** 2144–2153
10. Manjunatha, U. H., Madhusudan, K., Unniraman, S., Sikder, D., Chatterjee, M., Bhaduri, T., Radha, D. R., and Nagaraja, V. (2006) DNA topoisomerases from *Mycobacterium tuberculosis* and *Mycobacterium smegmatis. J. Ind. Inst. Sci.* **86,** 751–761
11. Zechiedrich, E. L., and Cozzarelli, N. R. (1995) Roles of topoisomerase IV and DNA gyrase in DNA unlinking during replication in *Escherichia coli. Genes Dev.* **9,** 2859–2869
12. Tretter, E. M., Schoeffler, A. J., Weisfield, S. R., and Berger, J. M. (2010) Crystal structure of the DNA gyrase GyrA N-terminal domain from *Mycobacterium tuberculosis. Proteins* **78,** 492–495
13. Fu, G., Wu, J., Liu, W., Zhu, D., Hu, Y., Deng, J., Zhang, X. E., Bi, L., and Wang, D. C. (2009) Crystal structure of DNA gyrase B′ domain sheds lights on the mechanism for T-segment navigation. *Nucleic Acids Res.* **37,** 5908–5916
14. Tretter, E. M., and Berger, J. M. (2012) Mechanisms for defining supercoiling set point of DNA gyrase orthologs. II. The shape of the GyrA subunit C-terminal domain (CTD) is not a sole determinant for controlling supercoiling efficiency. *J. Biol. Chem.* **287,** 18645–18654
15. Huang, Y. Y., Deng, J. Y., Gu, J., Zhang, Z. P., Maxwell, A., Bi, L. J., Chen, Y. Y., Zhou, Y. F., Yu, Z. N., and Zhang, X. E. (2006) The key DNA-binding residues in the C-terminal domain of *Mycobacterium tuberculosis* DNA gyrase A subunit (GyrA). *Nucleic Acids Res.* **34,** 5650–5659
16. Manjunatha, U. H., Maxwell, A., and Nagaraja, V. (2005) A monoclonal antibody that inhibits mycobacterial DNA gyrase by a novel mechanism. *Nucleic Acids Res.* **33,** 3085–3094
17. Karkare, S., Yousafzai, F., Mitchenall, L. A., and Maxwell, A. (2012) The role of Ca²⁺ in the activity of *Mycobacterium tuberculosis* DNA gyrase. *Nucleic Acids Res.* **40,** 9774–9787
18. Lall, N., and Meyer, J. J. (2000) Antibacterial activity of water and acetone extracts of the roots of *Euclea natalensis. J. Ethnopharmacol.* **72,** 313–316
19. Lall, N., and Meyer, J. J. (1999) *In vitro* inhibition of drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* by ethnobotanically selected South African plants. *J. Ethnopharmacol.* **66,** 347–354
20. Lall, N., and Meyer, J. J. (2001) Inhibition of drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis* by diospyrin, isolated from *Euclea natalensis. J. Ethnopharmacol.* **78,** 213–216
21. Pinto, A. V., and de Castro, S. L. (2009) The trypanocidal activity of naphthoquinones: a review. *Molecules* **14,** 4570–4590
22. van der Kooy, F., Meyer, J. J., and Lall, N. (2006) Antimycobacterial activity and possible mode of action of newly isolated neodiospyrin and other naphthoquinones from Euclea natalensis. *S. Afr. J. Botany* **72,** 349–352
23. Ray, S., Hazra, B., Mittra, B., Das, A., and Majumder, H. K. (1998) Diospyrin, a bisnaphthoquinone: a novel inhibitor of type I DNA topoisomerase of *Leishmania donovani. Mol. Pharmacol.* **54,** 994–999
24. Ting, C. Y., Hsu, C. T., Hsu, H. T., Su, J. S., Chen, T. Y., Tarn, W. Y., Kuo, Y. H., Whang-Peng, J., Liu, L. F., and Hwang, J. (2003) Isodiospyrin as a novel human DNA topoisomerase I inhibitor. *Biochem. Pharmacol.* **66,** 1981–1991
25. Mahapatra, A., Mativandlela, S. P., Binneman, B., Fourie, P. B., Hamilton, C. J., Meyer, J. J., van der Kooy, F., Houghton, P., and Lall, N. (2007) Activity of 7-methyljuglone derivatives against *Mycobacterium tuberculosis* and as subversive substrates for mycothiol disulfide reductase. *Bioorg. Med. Chem.* **15,** 7638–7646
26. Wang, H., Mao, Y., Chen, A. Y., Zhou, N., LaVoie, E. J., and Liu, L. F. (2001) Stimulation of topoisomerase II-mediated DNA damage via a mechanism involving protein thiolation. *Biochemistry* **40,** 3316–3323
27. Ali, J. A., Jackson, A. P., Howells, A. J., and Maxwell, A. (1993) The 43-kDa N-terminal fragment of the gyrase B protein hydrolyses ATP and binds coumarin drugs. *Biochemistry* **32,** 2717–2724
28. Maxwell, A., and Howells, A. J. (1999) in *DNA Topoisomerase Protocols. I. DNA Topology and Enzymes* (Bjornsti, M.-A., and Osheroff, N., eds) pp. 135–144, Humana Press, Totowa, NJ
29. Edwards, M. J., Flatman, R. H., Mitchenall, L. A., Stevenson, C. E., Le, T. B., Fiedler, H. P., McKay, A. R., Clarke, T. A., Buttner, M. J., Lawson, D. M., and Maxwell, A. (2009) A crystal structure of the bifunctional antibiotic, simocyclinone D8, bound to DNA gyrase. *Science* **326,** 1415–1418
30. Alt, S., Mitchenall, L. A., Maxwell, A., and Heide, L. (2011) Inhibition of DNA gyrase and DNA topoisomerase IV of *Staphylococcus aureus* and *Escherichia coli* by aminocoumarin antibiotics. *J. Antimicrob. Chemother.* **66,** 2061–2069
31. Edwards, M. J., Williams, M. A., Maxwell, A., and McKay, A. R. (2011) Mass spectrometry reveals that the antibiotic simocyclinone D8 binds to DNA gyrase in a "bent-over" conformation: evidence of positive cooperativity in binding. *Biochemistry* **50,** 3432–3440
32. Flatman, R. H., Howells, A. J., Heide, L., Fiedler, H.-P., and Maxwell, A. (2005) Simocyclinone D8: an inhibitor of DNA gyrase with a novel mode of action. *Antimicrob. Agents Chemother.* **49,** 1093–1100
33. Lindsey, R. H., Jr., Bromberg, K. D., Felix, C. A., and Osheroff, N. (2004) 1,4-Benzoquinone is a topoisomerase II poison. *Biochemistry* **43,** 7563–7574
34. Pan, X.-S., Ambler, J., Mehtar, S., and Fisher, L. M. (1996) Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae. Antimicrob. Agents Chemother.* **40,** 2321–2326
35. Khodursky, A. B., Zechiedrich, E. L., and Cozzarelli, N. R. (1995) Topoisomerase IV is a target of quinolones in *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* **92,** 11801–11805
36. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) Deciphering the biology of *Mycobacterium tubercu-*

losis from the complete genome sequence. *Nature* **393,** 537–544

37. Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T., and Tomizawa, J. (1977) Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 4772–4776

38. Sugino, A., Peebles, C. L., Kreuzer, K. N., and Cozzarelli, N. R. (1977) Mechanism of action of nalidixic acid: purification of *Escherichia coli nal*A gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 4767–4771

39. Willmott, C. J., and Maxwell, A. (1993) A single point mutation in the DNA gyrase A protein greatly reduces the binding of fluoroquinolones to the gyrase-DNA complex. *Antimicrob. Agents Chemother.* **37,** 126–127

40. Gormley, N. A., Orphanides, G., Meyer, A., Cullis, P. M., and Maxwell, A. (1996) The interaction of coumarin antibiotics with fragments of the DNA gyrase B protein. *Biochemistry* **35,** 5083–5092

41. Kampranis, S. C., and Maxwell, A. (1998) Conformational changes in DNA gyrase revealed by limited proteolysis. *J. Biol. Chem.* **273,** 22606–22614

42. Kampranis, S. C., Howells, A. J., and Maxwell, A. (1999) The interaction of DNA gyrase with the bacterial toxin CcdB: evidence for the existence of two gyrase-CcdB complexes. *J. Mol. Biol.* **293,** 733–744

43. Jacob, D. A., Mercer, S. L., Osheroff, N., and Deweese, J. E. (2011) Etoposide quinone is a redox-dependent topoisomerase II poison. *Biochemistry* **50,** 5660–5667

44. Wigley, D. B., Davies, G. J., Dodson, E. J., Maxwell, A., and Dodson, G. (1991) Crystal structure of an N-terminal fragment of the DNA gyrase B protein. *Nature* **351,** 624–629

45. Loo, J. A. (2000) Electrospray ionization mass spectrometry: a technology for studying noncovalent macromolecular complexes. *Int. J. Mass Spectrom.* **200,** 175–186

46. Kampranis, S. C., Gormley, N. A., Tranter, R., Orphanides, G., and Maxwell, A. (1999) Probing the binding of coumarins and cyclothialidines to DNA gyrase. *Biochemistry* **38,** 1967–1976

47. Wright, G. D. (2011) Molecular mechanisms of antibiotic resistance. *Chem. Comm.* **47,** 4055–4061

48. Livermore, D. M. (2009) Has the era of untreatable infections arrived? *J. Antimicrob. Chemother.* **64,** i29–36

49. Lewis, K., and Ausubel, F. M. (2006) Prospects for plant-derived antibacterials. *Nat. Biotechnol.* **24,** 1504–1507

50. Sibanda, T., and Okoh, A. I. (2007) The challenges of overcoming antibiotic resistance: plant extracts as potential sources of antimicrobial and resistance modifying agents. *Afr. J. Biotechnol.* **6,** 2886–2896

51. Cowan, M. M. (1999) Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **12,** 564–582

52. Bender, R. P., Lehmler, H. J., Robertson, L. W., Ludewig, G., and Osheroff, N. (2006) Polychlorinated biphenyl quinone metabolites poison human topoisomerase IIα: altering enzyme function by blocking the N-terminal protein gate. *Biochemistry* **45,** 10140–10152

53. Bender, R. P., Ham, A. J., and Osheroff, N. (2007) Quinone-induced enhancement of DNA cleavage by human topoisomerase IIα: adduction of cysteine residues 392 and 405. *Biochemistry* **46,** 2856–2864