

A monoclonal antibody that inhibits mycobacterial DNA gyrase by a novel mechanism

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ABSTRACT

DNA gyrase is a DNA topoisomerase indispensable for cellular functions in bacteria. We describe a novel, hitherto unknown, mechanism of specific inhibition of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* DNA gyrase by a monoclonal antibody (mAb). Binding of the mAb did not affect either GyrA–GyrB or gyrase–DNA interactions. More importantly, the ternary complex of gyrase–DNA–mAb retained the ATPase activity of the enzyme and was competent to catalyse DNA cleavage–religation reactions, implying a new mode of action different from other classes of gyrase inhibitors. DNA gyrase purified from fluoroquinolone-resistant strains of *M.tuberculosis* and *M.smegmatis* were inhibited by the mAb. The absence of cross-resistance of the drug-resistant enzymes from two different sources to the antibody-mediated inhibition corroborates the new mechanism of inhibition. We suggest that binding of the mAb in the proximity of the primary dimer interface region of GyrA in the heterotetrameric enzyme appears to block the release of the transported segment after strand passage, leading to enzyme inhibition. The specific inhibition of mycobacterial DNA gyrase with the mAb opens up new avenues for designing novel lead molecules for drug discovery and for probing gyrase mechanism.

INTRODUCTION

DNA topoisomerases are a group of enzymes that catalyse interconversions of different topological forms of DNA (1). DNA gyrase is a bacterial type II topoisomerase, which is able to supercoil DNA, a property not shared by other topoisomerases (1); the enzyme has now also been found in plants (2).

The mechanism of DNA supercoiling catalysed by gyrase involves a series of coordinated steps. The tetrameric holoenzyme (A_2B_2), formed by the association of two GyrA and GyrB subunits, binds duplex DNA to form a wrapped complex, in which one segment of DNA (the transported or ‘T’ segment) lies over another (the gate or ‘G’ segment) (3). The enzyme carries out transesterification reactions leading to a double-strand break in the G segment and simultaneous covalent attachment of the protein to the 5′ end of the cleaved duplex DNA. Following ATP binding, conformational changes in the enzyme pull the two ends of the cleaved G segment apart to open up a channel, allowing the T segment to pass into the enzyme. The T segment exits through the bottom gate of the enzyme, formed by the GyrA dimer, and hydrolysis of ATP sets up the initiation of the next supercoiling cycle.

The supercoiling reaction of DNA gyrase involves a series of complicated steps, which provide multiple opportunities to develop inhibitors. A number of inhibitors of diverse classes have been characterized (4); quinolones and coumarins are the most extensively studied. The quinolones are synthetic compounds, which interfere with the processes of rejoining the double-strand breaks in DNA. Newer quinolones, especially fluoroquinolones, have found wide applications clinically for a variety of bacterial infections (5). The coumarins are naturally occurring antibiotics, which inhibit the ATPase activity of gyrase (6). Cyclothialidines, a class of cyclic peptides, inhibit gyrase activity in a manner analogous to that of coumarins. In addition, two proteinaceous poisons, microcin B17 and CcdB, inhibit *Escherichia coli* gyrase in a manner similar to quinolones (4). More recently, a chromosomally encoded proteinaceous inhibitor of gyrase, GyrI, has been characterized (7,8). Most of these inhibitors fall into two groups based on their site of action and mechanism of inhibition: inhibitors such as fluoroquinolones, CcdB and microcin B17 affect the cleavage–religation step, while coumarins and cyclothialidines prevent ATP hydrolysis (4).

One-third of the global population is infected with tuberculosis with 6 million new cases reported each year; 20% of

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adult deaths and 6% of infant deaths are attributable to tuberculosis (9). Thus, *Mycobacterium tuberculosis* is the largest single infectious cause of mortality worldwide, killing ~2 million people annually (10). The synergy between tuberculosis and the AIDS epidemic (11), and the rapid rise in multidrug-resistant clinical isolates of *M.tuberculosis* have only reaffirmed tuberculosis as a major public health threat.

Studies on mycobacterial DNA gyrase and comparison of its properties with the *E.coli* enzyme have revealed many differences, which can potentially be exploited for tuberculosis therapy. For example, unlike the *E.coli* enzyme, *Mycobacterium smegmatis* gyrase is refractory to the plasmid-borne proteinaceous inhibitors CcdB and microcin B17, and exhibits reduced susceptibility to fluoroquinolones (12,13). Furthermore, *M.smegmatis* gyrase is more active as a decatenase than its *E.coli* counterpart. One strategy for the development of inhibitors of mycobacterial gyrase is to raise antibodies. Polyclonal antibodies raised against *M.tuberculosis* GyrA recognize GyrA proteins from other mycobacteria but not from *E.coli* (14). Monoclonal antibodies (mAbs) against the individual subunits of *M.smegmatis* gyrase have been raised and characterized (15,16). Two of these mAbs (C3 and H11) bind within the region between amino acids 351 and 415 of GyrA and have been shown to inhibit supercoiling by gyrase. A third antibody (E9) bound elsewhere and did not affect gyrase activity (15). In this paper, we have further investigated the mechanism of inhibition by one particular antibody, mAb:C3, and show that it inhibits the enzyme by a completely novel mechanism, which could be exploited to develop new agents for tuberculosis therapy.

MATERIALS AND METHODS

Bacterial strains and plasmids

M.smegmatis mc²155 and ciprofloxacin-resistant *M.smegmatis* strains (17) were obtained from P. K. Chakraborti (Institute of Microbial Technology, Chandigarh, India). *M.smegmatis* cultures was grown in modified Youmans and Karlson's medium with 0.2% Tween-80 (YK medium) at 37°C (18), and quinolone-resistant *M.smegmatis* was grown in YK medium containing 32 µg/ml ciprofloxacin. An ofloxacin-resistant *M.tuberculosis* clinical isolate was grown in Lowenstein Jensen medium at 37°C. Plasmid pJW312-SalI used in the purification of *E.coli* topoisomerase I was obtained from J. C. Wang (19).

Purification of DNA gyrase

M.smegmatis and *M.tuberculosis* DNA gyrase holoenzymes were purified by novobiocin–Sephrose column as described by Manjunatha *et al.* (16). Individual mycobacterial gyrase subunits were purified by immunoaffinity column chromatography (13). Fractions containing apparently homogeneous preparations as judged by SDS–PAGE were used for the experiments.

Immunoprecipitations, SDS–PAGE and western blotting

Immunoprecipitations were performed with mycobacterial gyrase (2 µg) with 15 µg/ml of mAb:C3, mAb:E9 or control

mouse IgG in potassium glutamate buffer as described previously (16). The samples were analysed by 8% SDS–PAGE and western blotting with GyrA- (1:20 000) and GyrB- (1:10 000) specific polyclonal antibodies. For immunoprecipitation of covalently closed circular DNA, the linearized pUC18 (BamHI) was end filled using labelled dNTPs and Klenow fragment of DNA polymerase I, followed by ligation using T4 DNA ligase. DNA gyrase was incubated with various concentrations of mAb before or after the addition of labelled DNA at 4°C for 1 h, followed by immunoprecipitation. The radioactive counts in the pellet and supernatant fractions were measured.

DNA supercoiling, relaxation and decatenation reactions

The supercoiling reactions were carried out as described previously (15). The IC₅₀ was defined as the concentration of the inhibitor that reduced the enzymatic activity observed with inhibitor-free controls by 50%. Relaxation and decatenation reactions were carried out as described previously (13). In relaxation assays, the enzyme was pre-incubated with 200 nM of mAb at 4°C for 15 min. Decatenation reactions were carried out in the presence of 500 ng of kDNA from *Leishmania donovani* (gift from H. K. Majumder) using 2 U of *M.smegmatis* enzyme along with 10 µg/ml ciprofloxacin, 20 µg/ml novobiocin, 20 µg/ml each of mAb:C3 and mAb:E9.

Electrophoretic mobility shift assay (EMSA)

EMSAs were carried out using a radiolabelled 240 bp DNA fragment encompassing the strong gyrase site from pBR322 (20). Labelled DNA (0.1 nM) was incubated with 5 nM mycobacterial gyrase in supercoiling buffer for 30 min at 4°C. For supershift assays, 5 nM of antibody was added either to the enzyme or to the DNA–enzyme complex. Unlabelled 240 bp DNA (5 and 10 times excess) was used in competition reactions. The samples were electrophoresed on a 3.5% native polyacrylamide gel at 4°C in 0.5× TBE buffer containing 10 mM MgCl₂.

ATPase reaction

ATPase assays were performed as described previously (21) with 10 U of purified *M.smegmatis* DNA gyrase with or without pre-incubation of 120 µg/ml mAb or control mouse IgG or 20 µg/ml novobiocin at 4°C for 30 min.

Cleavage and religation assays

Cleavage reactions (12.5 µl) were carried out in supercoiling buffer with linear radiolabelled 240 bp DNA fragment or negatively supercoiled DNA with varying concentrations of DNA gyrase in the presence of 30 µg ml⁻¹ ciprofloxacin as described previously (21). The samples were analysed on a 6% denaturing polyacrylamide gel or 1.2% agarose gel. For religation assays, the quinolone-induced cleavage reaction (after 15 min) was further incubated with 10 mM EDTA for 15 min at 37°C, followed by denaturation and proteinase K treatment.

Surface plasmon resonance

The mAb:GyrA:GyrB interaction was assessed on rabbit anti-mouse Fc antibody immobilized on a CM5 surface in a buffer containing 35 mM Tris–HCl (pH 7.6), 100 mM potassium

glutamate, 1 mM EDTA and 1 mM DTT. An aliquot of 20 nM MsGyrA:C3 IgG was passed across the surface, followed by 100 nM of purified GyrA. Subsequently, GyrB (100 nM) was allowed to interact and the GyrA–GyrB interaction was specifically disrupted in running buffer with 500 mM NaCl (16); 10 mM NaOH was used for surface regeneration. For measuring the mAb–GyrA binding affinities, the *M. smegmatis* GyrA was immobilized on the CM5 sensor chip and all measurements were carried out as described previously (16).

RESULTS

A GyrA-specific mAb inhibits DNA supercoiling, relaxation and decatenation by mycobacterial gyrase

In previous work, we showed that mAb:C3 (IgG₁), and its corresponding Fab fragment, inhibited *M. smegmatis* DNA gyrase supercoiling activity (15), whereas mAb:E9 (IgG₁) showed no effect on this reaction; neither antibody affected the supercoiling reaction of the *E. coli* enzyme. In the presence of stoichiometric amounts of mAb:C3 to *M. smegmatis* GyrA, complete inhibition of DNA supercoiling was observed (data not shown). In Figure 1A, we summarize the effects of C3 and E9 on the supercoiling reaction of *M. smegmatis* gyrase. We note a small increase in negative supercoiling in the presence of gyrase and C3, suggesting that limited strand passage may be possible in the presence of this antibody (see below).

The binding of mAb:C3 to DNA gyrase also inhibited the ATP-independent relaxation of negatively supercoiled DNA (Figure 1B); again no effect was seen with E9. Similar results were observed with Fab fragments (data not shown). Apart from intra-molecular strand passage leading to supercoiling or relaxation of DNA, gyrase also catalyses inter-molecular strand passage during the decatenation reaction (22); mycobacterial gyrase is an efficient decatenase (13). Therefore, the effect of mAb on the decatenation of catenated kinetoplast DNA was studied. As shown in Figure 1C, mAb:C3 also inhibited the decatenation activity of kinetoplast DNA to minicircles.

Effect of mAb on subunit assembly

Using surface plasmon resonance, we found the affinity of interaction (K_D) of mAb:C3, and its Fab fragment, with *M. smegmatis* GyrA to be 2.2×10^{-10} M and 1.7×10^{-10} M, respectively (data not shown), implying that the specific inhibition seen above is a consequence of a high-affinity interaction of mAb with gyrase. To address whether this high affinity binding of mAb:C3 to GyrA disrupts the GyrA dimer or GyrA–GyrB interactions, immunoprecipitation experiments were carried out. We found that mAb:C3 co-immunoprecipitated GyrB along with GyrA (Figure 2A), suggesting that the binding of the mAb does not disrupt subunit interaction. In contrast, another GyrA-specific antibody, MsGyrA:E9, which binds to different epitope masked in the holoenzyme, fails to immunoprecipitate the gyrase subunits (Figure 2A, lane 6) as observed previously (15). Surface plasmon resonance studies showed that when mAb:C3 was bound to the chip, GyrA could be bound and GyrB could then bind to the GyrA, confirming that the mAb-bound GyrA subunit interacts with GyrB (Figure 2B).

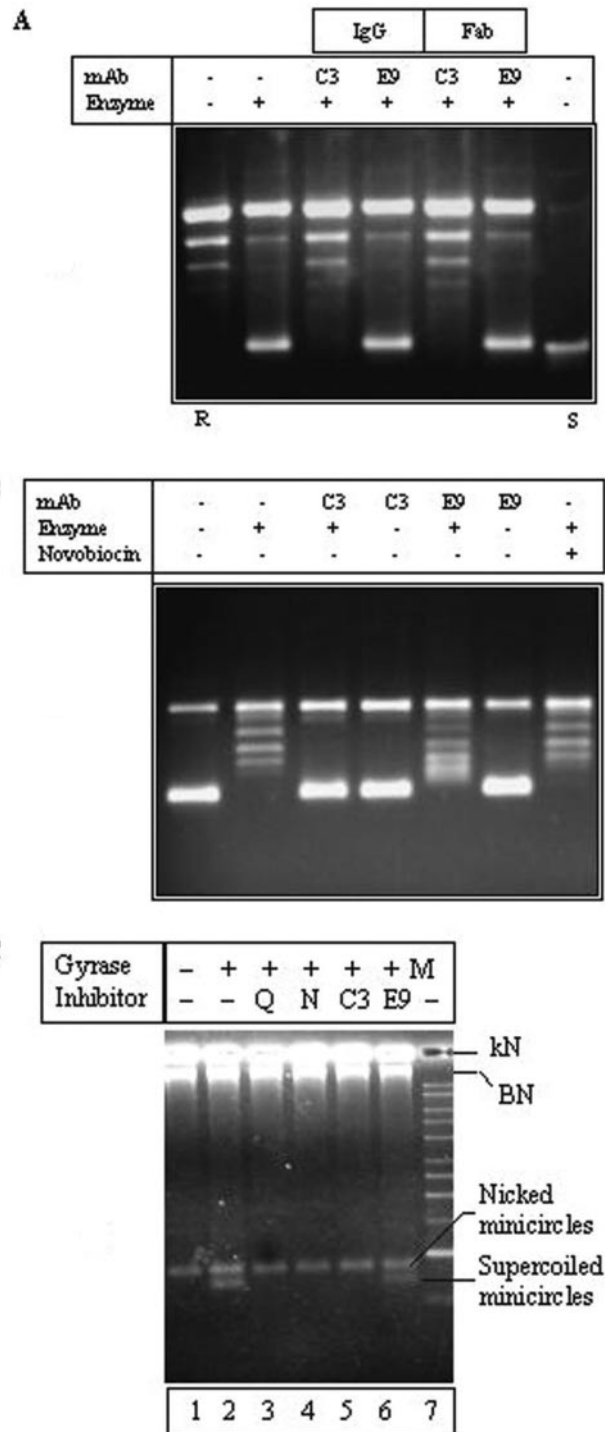


Figure 1. Inhibition of different enzymatic activities of DNA gyrase by mAb:C3. (A) Supercoiling: one unit of *M. smegmatis* DNA gyrase was pre-incubated with antibody (IgG or Fab as indicated), as described in Materials and Methods, and supercoiling assays were carried out using relaxed pUC18 as substrate. Samples were analysed on 1.2% agarose gels; R and S are relaxed and negatively supercoiled pUC18 DNA, respectively. (B) ATP-independent DNA relaxation: mAb was pre-incubated with gyrase and relaxation assays were carried out using supercoiled pUC18 DNA as substrate. Samples were analysed as described for the supercoiling assay. (C) Decatenation activity: reactions were carried out in the presence of kDNA (lane 1) with *M. smegmatis* enzyme (lane 2) along with ciprofloxacin (Q, lane 3), novobiocin (N, lane 4), mAb:C3 and E9 (C3 and E9, lanes 5 and 6, respectively). kN, kDNA network; BN, broken network.

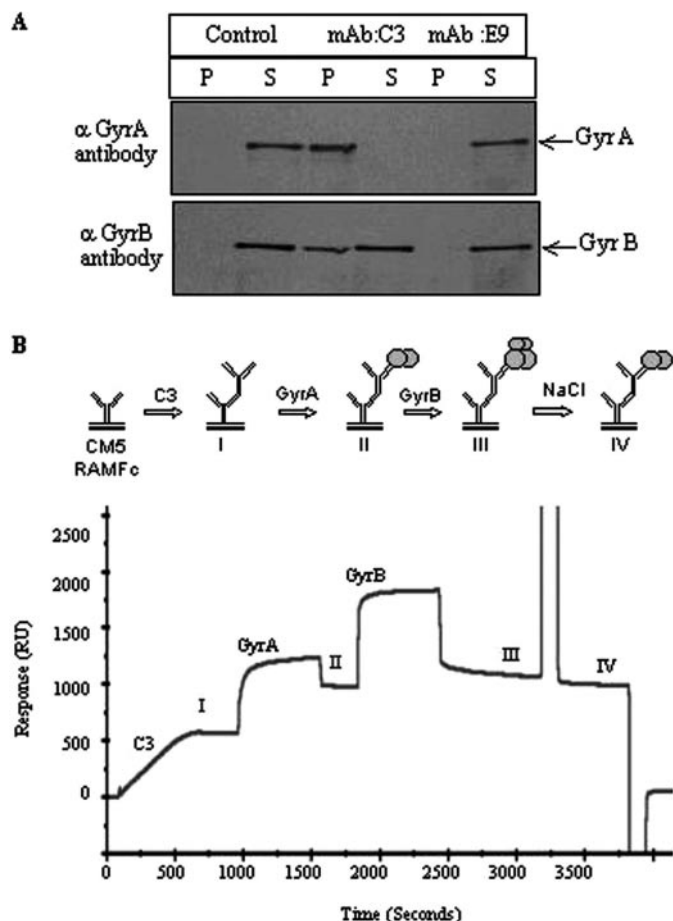


Figure 2. Effect of mAb:C3 on DNA gyrase subunit interaction. (A) Immunoprecipitation assay: Gyrase was immunoprecipitated with mAb:C3 or mAb:E9 or control mouse IgG. Immunocomplexes were recovered using protein G agarose. Both pellet (P) and supernatant (S) protein fractions were analysed by SDS-PAGE followed by western blotting with GyrA- or GyrB-specific polyclonal antibodies. (B) Surface plasmon resonance: a sensogram showing the interaction of gyrase subunits on rabbit α -mouse (RAM) Fc immobilized CM5 surface through mAb:C3. The various steps of interaction are shown schematically along with the bound response units. I: binding of C3 to α -mouse Fc; II: binding of GyrA to C3; III: binding of GyrB to GyrA; IV: dissociation of GyrB after salt wash.

These data suggest that mAb:C3 does not disrupt protein-protein interactions in gyrase.

Effect on DNA binding

EMSA and immunoprecipitation experiments were employed to evaluate the effect of mAb:C3 on gyrase-DNA interaction. Using EMSA, we found that mAb:C3 caused further retardation of the gyrase-DNA complex in a polyacrylamide gel, without release of free DNA (Figure 3A). A similar supershifted complex was also observed when Fab fragments were used in the assay instead of intact mAb (data not shown). However, control antibody showed no effect on gyrase-DNA interaction (Figure 3A). Because covalently closed circular DNA is the substrate for DNA supercoiling by gyrase, an immunoprecipitation reaction with such a substrate, mAb and gyrase was carried out. Formation of a gyrase-mAb-DNA ternary complex (Figure 3B) suggests that mAb:C3 neither inhibits gyrase subunit interaction nor inhibits DNA binding.

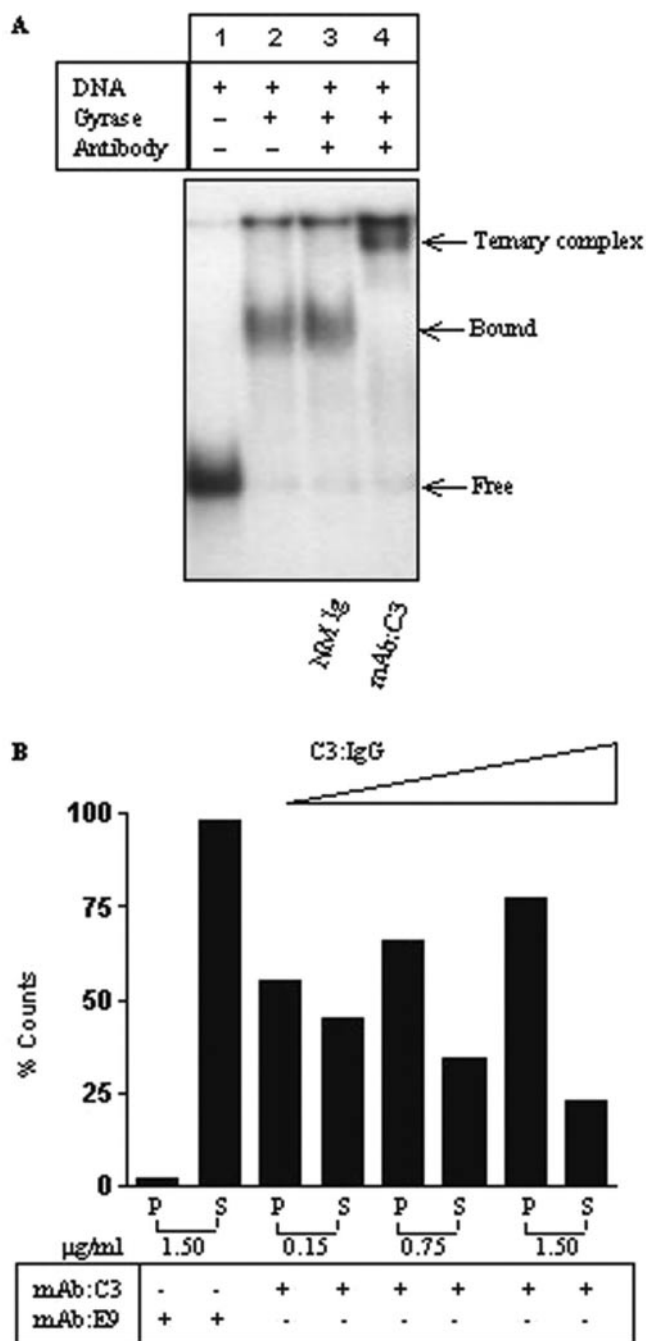


Figure 3. mAb binding to the gyrase-DNA complex. (A) EMSA with 240 bp labelled DNA fragment (lane 1) was carried out with gyrase (lane 2) incubated with control mouse immunoglobulin (NM Ig, lane 3) or gyrase with mAb:C3 (lane 4). The samples were electrophoresed on a 3.5% native polyacrylamide gel and autoradiographed. (B) Immunoprecipitation of covalently closed circular DNA. The enzyme was incubated with various concentrations of mAb (mAb:C3 or mAb:E9), followed by the addition of radiolabelled relaxed pUC18 DNA and immunoprecipitation was carried out. The DNA present in the GyrA-antibody complex (pellet) and the unbound (supernatant) were measured using liquid scintillation counter.

Binding of mAb does not block quinolone-stabilized DNA cleavage

In the preceding sections, we have shown that mAb:C3 does not affect subunit interaction or DNA binding by DNA gyrase.

Quinolone drugs stabilize gyrase in a particular conformation, such that the addition of SDS to the ternary complex of gyrase–DNA–quinolone results in DNA cleavage and reveals covalent attachment of the enzyme to the DNA (23,24). The ciprofloxacin-stabilized DNA cleavage reaction was used to assess the ability of the mAb to inhibit cleavage of the G segment. Pre-incubation of gyrase with inhibitory concentrations of mAb:C3 did not abolish quinolone-stabilized cleavage of either linear (Figure 4A) or negatively supercoiled DNA (Figure 4C). The amount of cleavable complex with the enzyme remained unaltered in the presence of saturating amounts of mAb (75 µg/ml) (Figure 4A and B); 3 µg/ml mAb is sufficient to completely inhibit supercoiling with 1 U of the enzyme (15). The DNA cleavage pattern remained unaltered in the presence or absence of mAb (Figure 4A). Furthermore, the data presented in Figure 4C, lane 9, indicate that mAb by itself did not induce or stimulate DNA cleavage by gyrase. Thus, the mechanism of inhibition of DNA gyrase by mAb is distinct from that of quinolones.

Limited proteolysis experiments (25) have shown that the formation of the gyrase–DNA–quinolone ternary complex results in large conformational changes in the enzyme, raising the possibility that mAb:C3 may fail to interact with quinolone-stabilized DNA gyrase. In order to address this possibility we examined whether mAb:C3 could still interact with a quinolone-stabilized gyrase–DNA complex using EMSA. While unlabelled DNA competed out non-covalent gyrase–DNA complexes (Figure 4D, lanes 3 and 4), the quinolone-stabilized complex could not be competed out, even with a large excess of DNA, indicating the formation of a stable, covalent cleavage complex (Figure 4D, lane 5). The appearance of a supershifted complex with the addition of the mAb (Figure 4D, lane 6) indicates that quinolone-induced conformational changes in the enzyme–DNA complex did not prevent mAb:C3 interaction with the enzyme.

The above results essentially demonstrate that G-segment binding and its subsequent cleavage by DNA gyrase were not inhibited by the mAb. DNA gyrase catalyses religation of the cleaved G segment after passage of the T segment through the DNA gate. The religation activity of gyrase can be assessed by adding EDTA to the gyrase–DNA–ciprofloxacin ternary complex (23,24,26). The binding of antibody to the gyrase–DNA–ciprofloxacin ternary complex had no effect on the reversibility of DNA cleavage (Figure 4E). EDTA by itself had no effect on GyrA–mAb interactions (data not shown). These results suggest that the mAb:C3 binding to gyrase does not affect the cleavage or religation steps of the supercoiling reaction.

Effect of mAb on ATPase activity of gyrase

ATP hydrolysis is essential for catalytic supercoiling by DNA gyrase. We found that mAb:C3 did not abolish the ATP hydrolysis activity of gyrase even when saturating amounts of mAb were used (Figure 5), whereas ATPase activity was completely inhibited by 5 µg/ml novobiocin. The mechanism of inhibition of gyrase by mAb:C3 is thus distinct from that of coumarins. The partial inhibition of the ATPase activity (Figure 5) may be significant and will be discussed below.

The effect of mAb:C3 on T-segment passage

Although the results in Figure 1 suggested that mAb:C3 inhibits supercoiling by DNA gyrase, there was evidence of low-level activity in the presence of the antibody. To explore this further, and address the effect of mAb on the strand-passage event, supercoiling assays were performed with a range of gyrase:DNA ratios in the presence of saturating amounts of mAb:C3. Under these conditions, we see further evidence of limited supercoiling in the presence of the antibody (Figure 6A, lanes 4–6), indicating that some strand passage of the T segment through the DNA–protein gate is possible under these conditions. At the higher enzyme concentrations, under these reaction conditions, we noted a slower moving species that was resistant to proteinase K digestion (Figure 6A, lanes 4–6). EM analysis of this species suggested that it is catenated DNA (data not shown). The appearance of catenated DNA, only at higher enzyme–DNA ratios, indicates that its formation might be a non-catalytic function of mAb-bound gyrase. In addition, the mAb-mediated catenane formation did not increase with time, suggesting that the reaction did not involve enzyme turnover. The formation of DNA catenanes is specific to mAb:C3 (both IgG as well as Fab) interaction with mycobacterial gyrase, as another mAb raised against mycobacterial gyrase, E9, had no effect (Figure 6A, lane 7); mAb:C3 did not affect *E.coli* gyrase activity (data not shown).

In the presence of the non-hydrolysable ATP analogue ADPNP (5'-adenylyl β,γ-imidodiphosphate), DNA gyrase can catalyse limited supercoiling (27). This result is consistent with nucleotide binding enabling one round of strand passage (28,29). In order to establish whether gyrase can catalyse limited strand passage in the presence of mAb:C3, we have examined the ADPNP reaction in the presence of this antibody (Figure 6B). The appearance of additional topoisomers in the presence of both ADPNP and mAb:C3 demonstrates that the mAb may permit a single-strand passage event (Figure 6B, lane 5). Supercoiling reactions in the presence of ADPNP alone showed a similar pattern (data not shown). These results together indicate that binding of the antibody allows limited strand-passage reaction.

Inhibition of quinolone-resistant DNA gyrase from *M.smegmatis* and *M.tuberculosis*

Since the inhibition of mycobacterial DNA gyrase supercoiling activity by mAb:C3 is by a mechanism very different from known modes of inhibition, the enzyme from quinolone-resistant strains should be susceptible to antibody-mediated inhibition. DNA gyrase from the ciprofloxacin-resistant *M.smegmatis* mc²155 strain (MIC₅₀ 64 µg/ml) was purified and used to test this prediction. The enzyme from the quinolone-sensitive strain showed an IC₅₀ of ~5 µg/ml for ciprofloxacin, whereas the enzyme from the resistant strain showed no inhibition even at 200 µg/ml (Figure 7A). DNA supercoiling activity was inhibited at 3 and 6 µg/ml of mAb:C3 for quinolone-sensitive and quinolone-resistant enzymes, respectively (Figure 7B), i.e. there is no apparent resistance to the antibody. DNA gyrase from the ofloxacin-resistant, highly virulent clinical isolate of *M.tuberculosis* (ICC-222) was also assayed for the effect of mAb. DNA gyrase from both the species of mycobacteria are similar in their size,

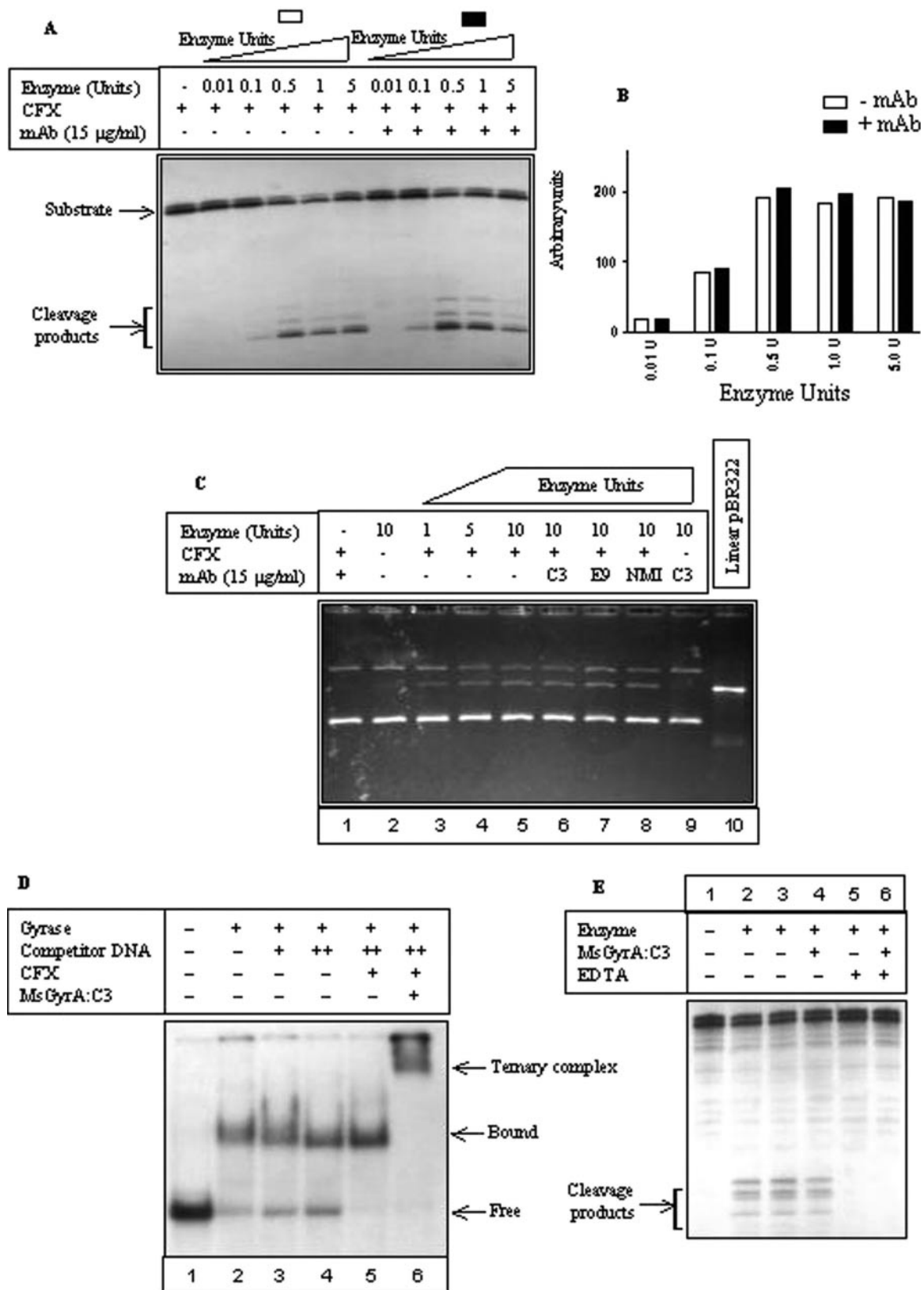


Figure 4. Effect of mAb on quinolone-stabilized cleavage and religation reactions. Cleavage reactions using a 240 bp labelled fragment of DNA (A and E) or negatively supercoiled pBR322 (C) as substrate in the presence of varying amounts of enzyme and different mAbs as indicated. (B) Quantitative representation of cleaved products of (A). (D) EMSA using 240 bp labelled fragment of DNA in the presence of 5× or 10× competitor unlabelled DNA and ciprofloxacin. (E) EDTA-induced religation reaction. A gyrase–CFX–DNA cleavage reaction (lane 2) was further incubated without (lanes 3 and 4) or with EDTA treatment (lanes 5 and 6). In lanes 4 and 6, the enzyme was pre-incubated with MsGyrA:C3.

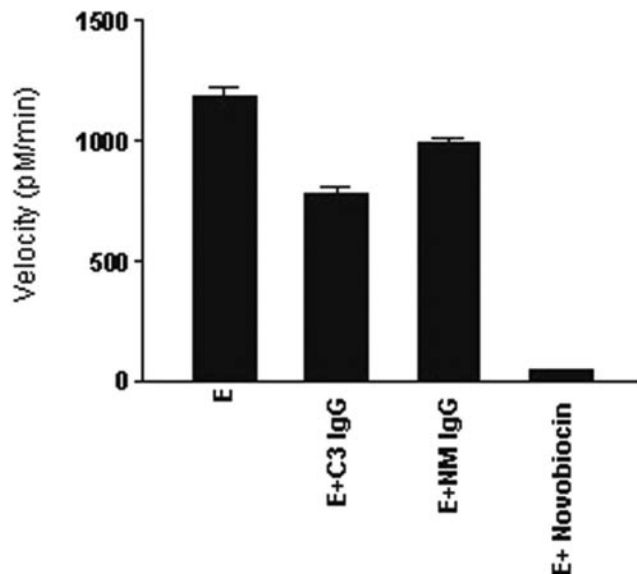


Figure 5. Effect of mAb on ATP hydrolysis. ATPase assays were carried out with 75 nM of *M. smegmatis* DNA gyrase in the presence of supercoiled pBR322 DNA. The enzyme (E) was pre-incubated in the presence of mAb:C3 (C3 IgG), control mouse IgG (NM IgG) or novobiocin, and ATPase reactions were carried out as described previously. The DNA-dependent ATPase rate is shown.

properties and antigenic cross reactivity (14–16). The purified enzyme has an IC_{50} of $\sim 10 \mu\text{g/ml}$ for ciprofloxacin, whereas the mAb:C3 inhibited the supercoiling activity at $3.0 \mu\text{g/ml}$, the same concentration required for the inhibition of *M. smegmatis* enzyme (Figure 7C). The absence of cross-resistance confirms the mode of action of mAb to be distinct to that of quinolones.

DISCUSSION

Most DNA gyrase inhibitors target only two different steps amongst many others during the supercoiling reaction. The high affinity, stoichiometric binding of mAb:C3 to GyrA suggests that its mode of inhibition is distinct. Binding of the mAb neither affected subunit interactions nor affected enzyme–DNA interactions, and a gyrase–holoenzyme DNA–mAb ternary complex is readily formed. The ternary complex retained DNA cleavage and religation activities. The conformational changes induced in the quinolone-stabilized cleavable complex (25) did not affect mAb binding. The antibody by itself did not arrest the reaction before the second transesterification, implying that the mode of action of mAb is distinct from that of quinolones. DNA gyrases from a quinolone-resistant *M. smegmatis* and *M. tuberculosis* were sensitive to mAb action, confirming the distinct mode of inhibition.

Furthermore, unlike coumarins, binding of mAb:C3 to mycobacterial gyrase did not abolish ATP hydrolysis by the enzyme. Cleavage of the G segment and capture of the T segment by the ATP-operated clamp is required to stimulate the ATPase activity of DNA gyrase (30,31). The retention of ATPase activity in the mAb–gyrase complex indicates that the presentation of the T segment to the ATP-operated clamp is not perturbed. The partial inhibition of the ATPase activity in the presence of C3 (Figure 5) may suggest some perturbation

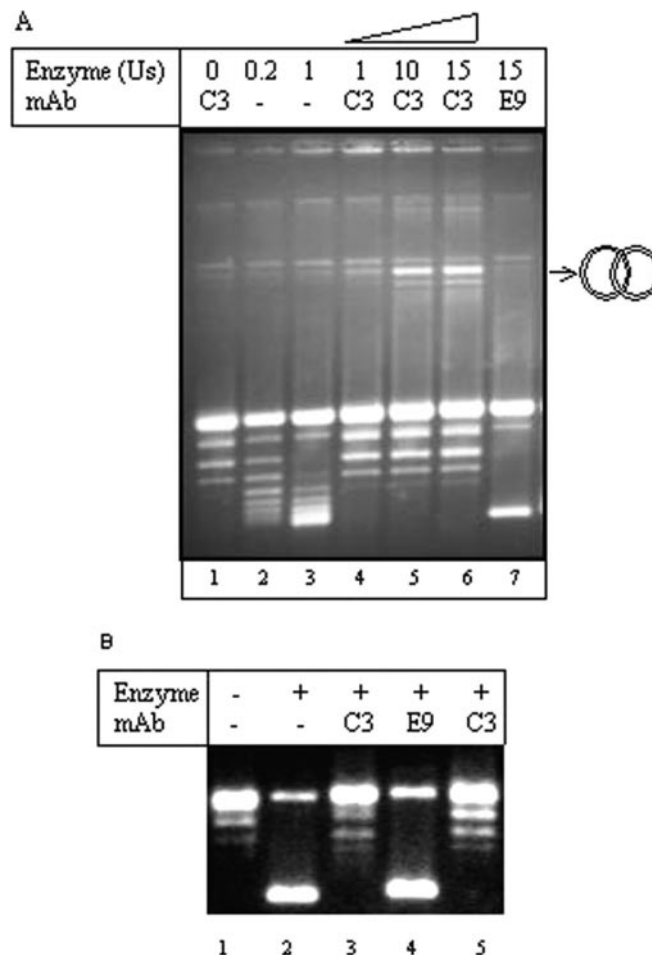


Figure 6. Effect of mAb on the strand-passage reaction. (A) The indicated amounts of enzyme (1 U = 25 fmol) were pre-incubated with 24 pmol of the mAb:C3 or mAb:E9 at 4°C for 15 min. An aliquot of 250 fmol of the relaxed DNA were added to the enzyme–antibody complex in supercoiling buffer and then incubated for 2 h at 37°C . SDS (0.16%) and proteinase K (90 $\mu\text{g/ml}$) were then added and the incubation continued for 30 min at 37°C . Samples were analysed on a 1.2% agarose gel electrophoresed at 25 V for 30 h. The slow migrating band in lanes 5 and 6 is thought to be a catenated species, as indicated. (B) Single round supercoiling. The assays are carried out as above with 15 U of enzyme (lanes 2–5). Lane 1, no enzyme; lane 5, 0.5 mM ADPNP is present along with mAb:C3.

of the strand-passage reaction in the presence of the antibody (see below).

A conformation-dependent epitope, comprising amino acids 351 and 415 of GyrA, is recognized by the mAb (15). Sequence alignment with the *E. coli* GyrA subunit revealed that this stretch corresponds to amino acid residues between 340 and 402 of *E. coli* GyrA. These residues form a long helix ($\alpha 14$), positioned in the proximity of the primary dimer interface region. The structure is present in yeast topoisomerase II (32) and seems to be conserved in all other type II topoisomerases. From the sequence comparison and secondary structure prediction, the mAb interacting domain of mycobacterial gyrase appears to form a similar structure. The conserved long alpha helix is an integral part in the formation of the second gate for the exit of the T segment during catalysis (33). The binding of the mAb proximal to the bottom dimer interface region could effectively block the exit gate.

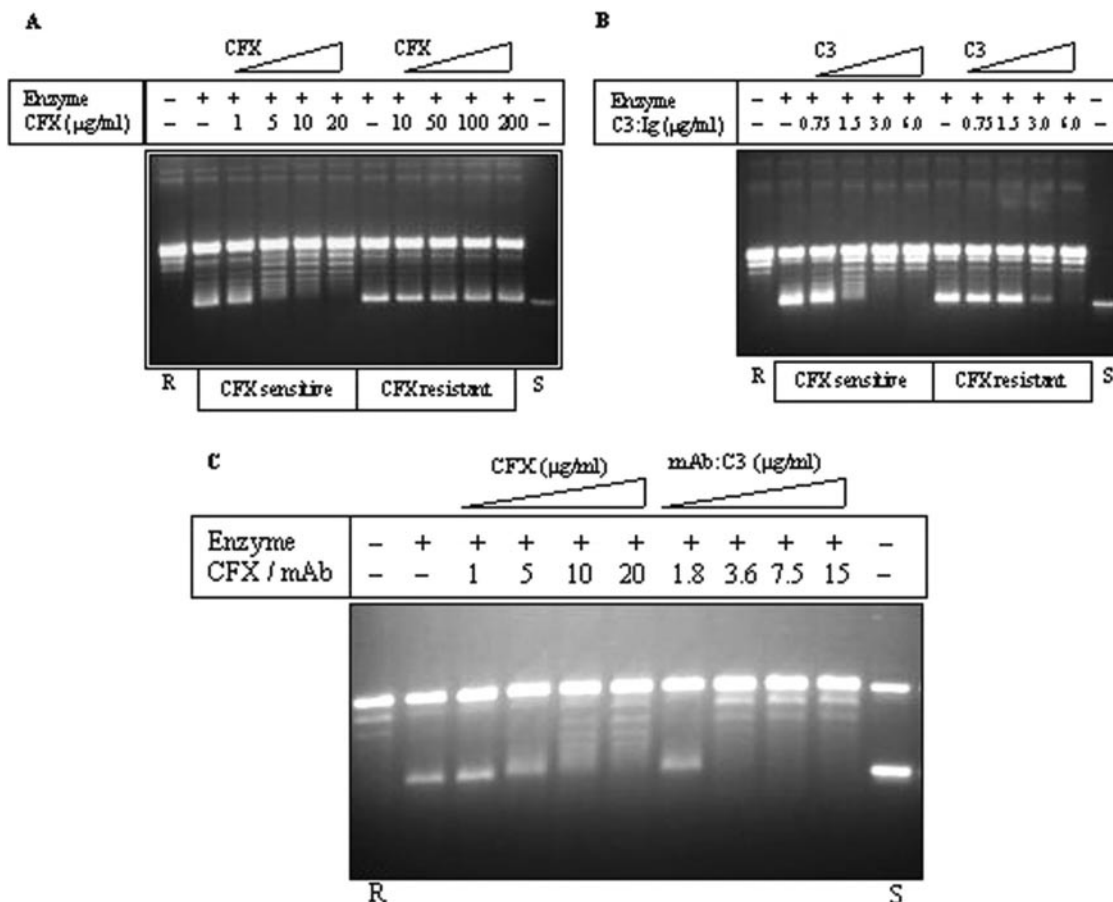


Figure 7. Inhibition of DNA gyrase from quinolone-resistant mycobacteria. Supercoiling reactions were carried out in the presence of varying concentrations of ciprofloxacin (CFX) (A) or mAb:C3 (B), with CFX-sensitive or CFX-resistant enzymes, isolated from wild-type and drug-resistant *M. smegmatis*, as indicated. R and S are relaxed and negatively supercoiled pUC18 DNA, respectively. (C) Supercoiling reactions carried out with DNA gyrase isolated from an ofloxacin-resistant *M. tuberculosis* strain, in the presence of CFX and mAb:C3, as indicated.

Based on the data in this paper, we propose a model for the interaction of mAb:C3 with GyrA and how this interaction inhibits DNA supercoiling (Figure 8). Supercoiling by gyrase involves wrapping of DNA around the enzyme such that the T segment lies over the G segment (3). Cleavage of the G segment allows passage of the T segment through the break and into the enzyme. The T segment is released by the opening of the exit gate [primary dimer interface (33)]. The epitope for mAb:C3 lies near this interface. Binding of the Ab to this region and blocking of the exit gate could account for the results we have observed. Such a mode of action would neither affect protein–protein and protein–DNA interactions by gyrase nor would it affect DNA cleavage–religation by the enzyme; in this sense, the action of mAb:C3 resembles the effect of cross-linking the exit gate of gyrase (34) and yeast topoisomerase II (35). Blocking of the exit gate would be expected to inhibit all strand-passage reactions of gyrase, as we have observed (Figure 1). The evidence of limited supercoiling (Figures 1A and 6) would be consistent with the mAb blocking step III of the supercoiling cycle (Figure 8) and just allowing one cycle of strand passage, again consistent with what has been observed in cross-linking experiments (34). DNA-dependent ATP hydrolysis requires binding of both the G and the T segments to gyrase and would not necessarily

be expected to be affected by the Ab. However, the low-level inhibition seen (Figure 5) might be a consequence of the failure of the enzyme to release the T segment; very similar observations were made with enzyme cross-linked at the exit gate (34). This proposed mode of inhibition is very different from that of known antibiotics.

The results highlight the opportunities to exploit DNA gyrase as a drug target relying on novel strategies. Antibody mAb:C3 cross-reacts with GyrA from other mycobacteria [*Mycobacterium bovis*, *Mycobacterium leprae* and *Mycobacterium avium* (15)] but not with the *E. coli* enzyme, it thus opens up the avenues for strategic design of specific lead molecules targeted against mycobacterial infections. This is in contrast to quinolones, coumarins and cyclothialidines, which inhibit DNA gyrases from many bacterial species. Antigen binding by peptide sequences from selected complementarity determining regions of mAbs have been demonstrated to have specificities similar to those of the original antibody molecule (36,37). These examples form the basis for the design of peptides that would specifically inhibit DNA gyrase from mycobacteria. The ability of small molecules to selectively stabilize a transient dimer interface within a type II DNA topoisomerase has been demonstrated previously. Classen *et al.* (38) showed that ICRF-187 binding to topoisomerase

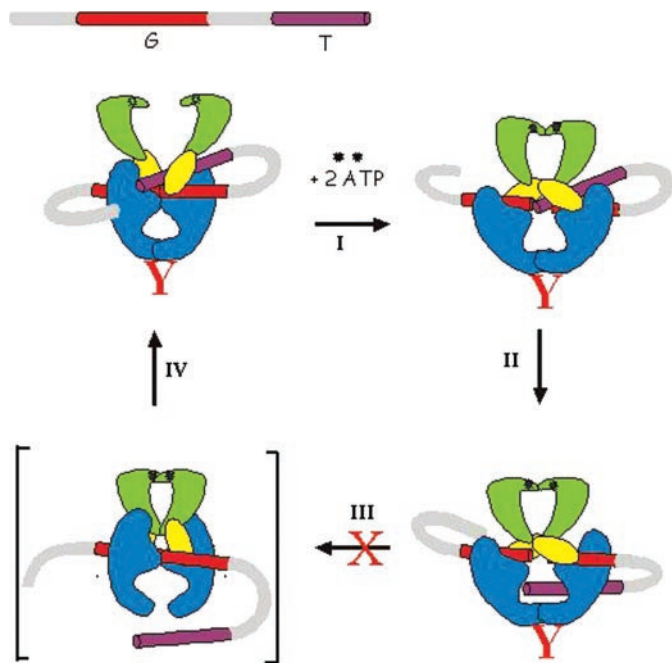


Figure 8. Model for the interaction of mAb:C3 with DNA gyrase. The different steps of the supercoiling reaction are depicted, based on the proposed model for DNA gyrase (39,40). The GyrA N-terminal domain is shown in blue, and the GyrB N- and C-terminal domains are shown in green and yellow, respectively. The C-terminal domain of GyrA is not shown for clarity. The solid bar (grey) represents double-stranded DNA with the segment of DNA that is cleaved (G segment) in red and the segment of DNA that is transported through the enzyme (T segment) in purple. Gyrase transiently cleaves the G segment and transports the T segment through this break before its religation. mAb:C3 (shown as red 'Y'-shaped molecule) is proposed to interact with GyrA near the exit gate such as to prevent the release of the T segment (step III).

II stabilizes ATP-bound dimerized state preventing enzyme turnover. The absence of cross-resistance to fluoroquinolone-resistant DNA gyrase by the mAb warrants the pursuit of this strategy further as it could aid in counteracting drug resistance problems. In addition, these new inhibitors would be invaluable tools in elucidating various steps of the supercoiling reaction, which in turn would facilitate the rational design of lead molecules.

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REFERENCES

1. Champoux, J.J. (2001) DNA topoisomerases: structure, function, and mechanism. *Annu. Rev. Biochem.*, **70**, 369–413.
2. Wall, M.K., Mitchenall, L.A. and Maxwell, A. (2004) *Arabidopsis thaliana* DNA gyrase is targeted to chloroplasts and mitochondria. *Proc. Natl Acad. Sci. USA*, **101**, 7821–7826.
3. Hedde, J.G., Mittelheiser, S., Maxwell, A. and Thomson, N.H. (2004) Nucleotide binding to DNA gyrase causes loss of DNA wrap. *J. Mol. Biol.*, **337**, 597–610.
4. Maxwell, A. (1997) DNA gyrase as a drug target. *Trends Microbiol.*, **5**, 102–109.
5. Drlica, K. and Malik, M. (2003) Fluoroquinolones: action and resistance. *Curr. Top. Med. Chem.*, **3**, 249–282.
6. Maxwell, A. and Lawson, D.M. (2003) The ATP-binding site of type II topoisomerases as a target for antibacterial drugs. *Curr. Top. Med. Chem.*, **3**, 283–303.
7. Nakanishi, A., Oshida, T., Matsushita, T., Imajoh-Ohmi, S. and Ohnuki, T. (1998) Identification of DNA gyrase inhibitor (GyrI) in *Escherichia coli*. *J. Biol. Chem.*, **273**, 1933–1938.
8. Chatterji, M. and Nagaraja, V. (2002) GyrI: a counter-defensive strategy against proteinaceous inhibitors of DNA gyrase. *EMBO Rep.*, **3**, 261–267.
9. Dye, C., Scheele, S., Dolin, P., Pathania, V. and Ravigliione, M.C. (1999) Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA*, **282**, 677–686.
10. Bloom, B.R. and Small, P.M. (1998) The evolving relation between humans and *Mycobacterium tuberculosis*. *N. Engl. J. Med.*, **338**, 677–678.
11. Kaufmann, S.H. and van Embden, J.D. (1993) Tuberculosis: a neglected disease strikes back. *Trends Microbiol.*, **1**, 2–5.
12. Chatterji, M., Unniraman, S., Mahadevan, S. and Nagaraja, V. (2001) Effect of different classes of inhibitors on DNA gyrase from *Mycobacterium smegmatis*. *J. Antimicrob. Chemother.*, **48**, 479–485.
13. 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.
14. Manjunatha, U.H., Madhusudan, K., Visweswariah, S.S. and Nagaraja, V. (2000) Structural heterogeneity in DNA gyrases from Gram-positive and Gram-negative bacteria. *Curr. Sci.*, **79**, 968–974.
15. Manjunatha, U.H., Mahadevan, S., Visweswariah, S.S. and Nagaraja, V. (2001) Monoclonal antibodies to mycobacterial DNA gyrase A inhibit DNA supercoiling activity. *Eur. J. Biochem.*, **268**, 2038–2046.
16. Manjunatha, U.H., Somesh, B.P., Nagaraja, V. and Visweswariah, S.S. (2001) A *Mycobacterium smegmatis* gyrase B specific monoclonal antibody reveals association of gyrase A and B subunits in the cell. *FEMS Microbiol. Lett.*, **194**, 87–92.
17. Banerjee, S.K., Bhatt, K., Rana, S., Misra, P. and Chakraborti, P.K. (1996) Involvement of an efflux system in mediating high level of fluoroquinolone resistance in *Mycobacterium smegmatis*. *Biochem. Biophys. Res. Commun.*, **226**, 362–368.
18. Nagaraja, V. and Gopinathan, K.P. (1980) Requirement for calcium ions in mycobacteriophage I3 DNA injection and propagation. *Arch. Microbiol.*, **124**, 249–254.
19. Lynn, R.M. and Wang, J.C. (1989) Peptide sequencing and site-directed mutagenesis identify tyrosine-319 as the active site tyrosine of *Escherichia coli* DNA topoisomerase I. *Proteins*, **6**, 231–239.
20. Fisher, L.M., Mizuuchi, K., O'Dea, M.H., Ohmori, H. and Gellert, M. (1981) Site-specific interaction of DNA gyrase with DNA. *Proc. Natl Acad. Sci. USA*, **78**, 4165–4169.
21. Chatterji, M., Unniram, S., Maxwell, A. and Nagaraja, V. (2000) The additional 165 amino acids in the B protein of *Escherichia coli* DNA gyrase have an important role in DNA binding. *J. Biol. Chem.*, **275**, 22888–22894.
22. Kreuzer, K.N. and Cozzarelli, N.R. (1980) Formation and resolution of DNA catenanes by DNA gyrase. *Cell*, **20**, 245–254.
23. 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. USA*, **74**, 4772–4776.
24. Sugino, A., Peebles, C.L., Kreuzer, K.N. and Cozzarelli, N.R. (1977) Mechanism of action of nalidixic acid: purification of *Escherichia coli* nalA gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. *Proc. Natl Acad. Sci. USA*, **74**, 4767–4771.

25. Kampranis,S.C. and Maxwell,A. (1998) Conformational changes in DNA gyrase revealed by limited proteolysis. *J. Biol. Chem.*, **273**, 22606–22614.
26. Kreuzer,K.N. and Cozzarelli,N.R. (1979) *Escherichia coli* mutants thermosensitive for deoxyribonucleic acid gyrase subunit A: effects on deoxyribonucleic acid replication, transcription, and bacteriophage growth. *J. Bacteriol.*, **140**, 424–435.
27. Tamura,J.K., Bates,A.D. and Gellert,M. (1992) Slow interaction of 5'-adenylyl β,γ -imidodiphosphate with *Escherichia coli* DNA gyrase. *J. Biol. Chem.*, **267**, 9214–9222.
28. Sugino,A., Higgins,N.P., Brown,P.O., Peebles,C.L. and Cozzarelli,N.R. (1978) Energy coupling in DNA gyrase and the mechanism of action of novobiocin. *Proc. Natl Acad. Sci. USA*, **75**, 4838–4842.
29. Bates,A.D., O'Dea,M.H. and Gellert,M. (1996) Energy coupling in *Escherichia coli* DNA gyrase: the relationship between nucleotide binding, strand passage, and DNA supercoiling. *Biochemistry*, **35**, 1408–1416.
30. Tingey,A.P. and Maxwell,A. (1996) Probing the role of the ATP-operated clamp in the strand-passage reaction of DNA gyrase. *Nucleic Acids Res.*, **24**, 4868–4873.
31. Kampranis,S.C., Bates,A.D. and Maxwell,A. (1999) A model for the mechanism of strand passage by DNA gyrase. *Proc. Natl Acad. Sci. USA*, **96**, 8414–8419.
32. Berger,J.M., Gamblin,S.J., Harrison,S.C. and Wang,J.C. (1996) Structure at 2.7 Å resolution of a 92K yeast DNA topoisomerase II fragment. *Nature*, **379**, 225–232.
33. Morais Cabral,J.H., Jackson,A.P., Smith,C.V., Shikotra,N., Maxwell,A. and Liddington,R.C. (1997) Structure of the DNA breakage-reunion domain of DNA gyrase. *Nature*, **388**, 903–906.
34. Williams,N.L. and Maxwell,A. (1999) Locking the DNA gate of DNA gyrase: investigating the effects on DNA cleavage and ATP hydrolysis. *Biochemistry*, **38**, 14157–14164.
35. Roca,J., Berger,J.M., Harrison,S.C. and Wang,J.C. (1996) DNA transport by a type II topoisomerase: direct evidence for a two-gate mechanism. *Proc. Natl Acad. Sci. USA*, **93**, 4057–4062.
36. Monnet,C., Laune,D., Laroche-Traineau,J., Biard-Piechaczyk,M., Briant,L., Bes,C., Pugniere,M., Mani,J.C., Pau,B., Cerutti,M. *et al.* (1999) Synthetic peptides derived from the variable regions of an anti-CD4 monoclonal antibody bind to CD4 and inhibit HIV-1 promoter activation in virus-infected cells. *J. Biol. Chem.*, **274**, 3789–3796.
37. Zanetti,M., Filaci,G., Lee,R.H., del Guercio,P., Rossi,F., Bacchetta,R., Stevenson,F., Barnaba,V. and Billetta,R. (1993) Expression of conformationally constrained adhesion peptide in an antibody CDR loop and inhibition of natural killer cell cytotoxic activity by an antibody antigenized with the RGD motif. *EMBO J.*, **12**, 4375–4384.
38. Classen,S., Olland,S. and Berger,J.M. (2003) Structure of the topoisomerase II ATPase region and its mechanism of inhibition by the chemotherapeutic agent ICRF-187. *Proc. Natl Acad. Sci. USA*, **100**, 10629–10634.
39. Costenaro,L., Grossmann,J.G., Ebel,C. and Maxwell,A. (2005) Small-angle X-ray scattering reveals the solution structure of the full-length DNA gyrase A subunit. *Structure*, **13**, 287–296.
40. Pierrat,O.A. and Maxwell,A. (2005) Evidence for the role of DNA strand passage in the mechanism of action of microcin B17 on DNA gyrase. *Biochemistry*, **44**, 4204–4215.