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Effect of different classes of inhibitors on DNA gyrase from *Mycobacterium smegmatis*

Monalisa Chatterji, Shyam Unniraman, Sethuraman Mahadevan and Valakunja Nagaraja*

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India 560012

Quinolones, coumarins, cyclothialidines, CcdB and microcin B17 inhibit DNA gyrase. Information regarding these various inhibitors comes from studies performed with the enzyme from *Escherichia coli*, and subsequent analyses have also primarily been confined to this system. We have carried out a detailed analysis of the effect of various groups of inhibitors on *Mycobacterium smegmatis* gyrase and demonstrate differential susceptibility of the *E. coli* and *M. smegmatis* gyrases. Interestingly, *M. smegmatis* gyrase was refractory to the plasmid-borne proteinaceous inhibitors CcdB and microcin B17. Ciprofloxacin, a fluoroquinolone, showed a 10-fold reduction in efficacy against *M. smegmatis* compared with *E. coli* gyrase. We have also shown that etoposide, an antineoplastic drug, inhibits DNA gyrase activity by trapping the gyrase–DNA complex. DNA gyrases from both *E. coli* and *M. smegmatis* were susceptible to etoposide at comparable levels.

Introduction

DNA gyrase, a type II DNA topoisomerase, is the only known enzyme that negatively supercoils DNA in the presence of ATP.[1,2] In addition, the enzyme catenates–decatenates double-stranded DNA circles, resolves knots in DNA and also relaxes negatively supercoiled DNA in the absence of ATP. As a result, the enzyme is vital for almost all cellular processes that involve duplex DNA, namely replication, recombination and transcription. It is exclusive to the prokaryotic kingdom and is essential for the survival of the organism. Thus, DNA gyrase appears to be an ideal target for antibacterial drugs.

DNA gyrase cleaves a double strand, passes another duplex through it and reseals it.[2,3] Extensive biochemical characterization of the enzyme from *Escherichia coli* has demonstrated that the active enzyme is a heterotetramer composed of GyrA and GyrB. The N-terminal two-thirds of GyrA harbours the cleavage–religation activity. The C-terminal one-third is responsible for wrapping DNA around itself in a positive superhelical sense. In addition, the N-terminal half of GyrB hydrolyses ATP, and the C-terminal half is involved in binding to GyrA and DNA. A variety of inhibitors have been found to interfere with specific enzymic reactions of DNA gyrase, rendering it inactive.[4–6] Two major families of compounds that inhibit *E. coli* DNA gyrase are quinolones and coumarins. Other gyrase inhibitors include ribosomally synthesized proteinaceous poisons like microcin B17, CcdB and cyclic peptide cyclothialidines.

Coumarins and cyclothialidines are naturally occurring compounds produced by certain species of *Streptomyces*.[7,8] Both classes of molecule compete with ATP for binding to GyrB, and thus inhibit the ATPase activity of the enzyme.[9,10] In contrast, quinolones and fluoroquinolones are synthetic compounds and specific members of this family preferentially inhibit either prokaryotic or eukaryotic type II topoisomerases.[11,12] These compounds stabilize the reversible enzyme–DNA covalent intermediate,[13,14] leading to the generation of double-stranded breaks in DNA. The protein–DNA adducts thus generated also act as blocks for DNA tracking enzymes like RNA and DNA polymerases.[15] Point mutations conferring resistance to quinolones primarily map to the N-terminal region of GyrA whereas a few map to the C-terminal half of GyrB.[6]

CcdB and microcin B17 are both plasmid-encoded proteinaceous inhibitors produced by Gram-negative bacteria.[16,17] CcdB has been shown to bind to GyrA of *E. coli* and stabilize the gyrase–DNA complex in a manner reminiscent of, but not identical to, the quinolones.[18] In agreement with this, mutations conferring resistance to CcdB map to GyrA.[19] Like CcdB, microcin B17 also acts by stabilizing the gyrase–DNA covalent complex; however, the exact mode of inhibition remains to be elucidated.[20] In contrast

*Corresponding author. Tel: +91-80-360-0668; Fax: +91-80-360-2697; E-mail: vraj@mcbl.iisc.ernet.in

to CcdB and quinolones, the only mutation in *E. coli* conferring resistance to microcin B17 maps to Trp-751 in GyrB.

Information regarding various inhibitors of DNA gyrase comes primarily from studies performed with the enzyme from *E. coli*. Although DNA gyrase is a relatively conserved protein, analysis of the primary sequence of *gyr* genes from different organisms reveals considerable divergence between Gram-positive and Gram-negative bacteria.[21,22] These differences appear to extend to the domainal organization of the protein as well.[23] Furthermore, based on the sequence of emergence of resistant mutants in Gram-negative bacteria, DNA gyrase is believed to be the primary site of quinolone action, whereas topoisomerase IV is the secondary target.[12,24] In contrast, DNA gyrase in Gram-positive bacteria is intrinsically less susceptible to quinolones and the primary target in these organisms appears to be topoisomerase IV. Thus, it appears that DNA gyrase from various organisms exhibits differential behaviour towards inhibitors. Therefore, a thorough evaluation of the effects of a range of inhibitors on DNA gyrase from a Gram-positive bacterium is imperative.

Infections caused by mycobacteria are the single largest cause of death worldwide. Fluoroquinolones have been used with limited success as part of a second-line chemotherapeutic regime against mycobacterial diseases. With the global emergence of multidrug-resistant tuberculosis, there is an urgent need to develop new anti-mycobacterials. A study of the efficacy of known inhibitors against the mycobacterial enzyme would facilitate the design of new inhibitors with greater specificity. We have analysed the susceptibility of DNA gyrase from *Mycobacterium smegmatis* to inhibitors known to act against the *E. coli* enzyme. *M. smegmatis* has been developed as a model system to understand both the basic metabolism and the drug susceptibility of mycobacteria.[25–27] The gyrase subunits from *M. smegmatis* are >90% similar (GyrA, 93.7%; GyrB, 92%) to those present in *M. tuberculosis* at the amino acid level.[21] Our study reveals varied susceptibility of the *M. smegmatis* enzyme to different classes of gyrase inhibitor.

Materials and methods

Bacterial strains and plasmids

M. smegmatis SN2 cells were used for purification of DNA gyrase. pPH3, pAG111[28] and pJW312-*Sal*I[29] were used to overexpress *E. coli* GyrA, GyrB and topoisomerase I, respectively. *E. coli* strains ZK4 and ZK650 were the microcin B17 susceptible and producer strains, respectively.[30]

Inhibitors

Novobiocin and ciprofloxacin (Sigma, St Louis, MO, USA) were dissolved in water and 0.1 M NaOH, respectively. Etoposide (Sigma) and cyclothialidine (gift from E. Goetschi, F. Hoffmann-La Roche Ltd, Basel, Switzerland) were dissolved in 10% (v/v) dimethyl sulfoxide (DMSO). Lyophilized CcdB was reconstituted in supercoiling reaction buffer. Microcin B17 was purified from ZK650 cells. Acid-soluble lysates were prepared by boiling stationary phase cells in 100 mM acetic acid containing 1 mM EDTA. After neutralization, the extract was loaded on SepPak C18 column (Waters, Milford, MA, USA). The column was washed sequentially with 10% and 20% ethanol, then microcin B17 was eluted with 30% ethanol. The antibacterial activity of the preparation was determined as described previously.[31]

Enzymes and substrate preparation

E. coli GyrA and GyrB were purified as described previously.[32] *M. smegmatis* gyrase was purified as described previously,[25,33] with certain modifications. Cells were grown in modified Youman and Karlson's medium[34] to mid-log phase (12–14 h of growth) and harvested. The pellet was subsequently resuspended in TGEM [50 mM Tris–HCl pH 7.5, 5% (v/v) glycerol, 1 mM EDTA and 2 mM β-mercaptoethanol], sonicated and centrifuged at 100 000**g** for 90 min. The supernatant (S_{100}) was subjected to an ammonium sulphate fractionation (70% saturation). The pellet was dissolved in and dialysed against TGEM, and loaded on to a novobiocin–Sepharose column. The column was washed with TGEM and the holoenzyme was eluted with 5 M urea. The proteins were renatured by step dialysis against TGEM containing 4, 3, 2, 1 and 0 M urea. The proteins were stored in TGEM containing 100 mM potassium glutamate. Specific activity of purified DNA gyrases was calculated, with 1 U defined as the amount of enzyme required to completely supercoil 500 ng of relaxed pUC18 DNA at 37°C in 30 min. *E. coli* topoisomerase I was purified from DH10B cells harbouring the pJW312-*Sal*I plasmid. The S_{100} preparation and ammonium sulphate precipitation were carried out as described for DNA gyrase. The pellet was redissolved and dialysed against TGEM and was loaded on a Hi-Trap heparin column (Amersham Pharmacia Biotech, Uppsala, Sweden) and eluted with a 0–1 M NaCl gradient. Supercoiled pUC18 and pBR322 were prepared by standard DNA purification protocols.[35] Relaxed pUC18 was prepared by incubating supercoiled DNA with *E. coli* topoisomerase I in 40 mM Tris–HCl pH 8, 1 mM EDTA, 5 mM $MgCl_2$ and 40 mM NaCl for 1 h at 30°C.

Enzyme assays

Supercoiling assays were carried out by incubating 500 ng of relaxed pUC18 at 37°C in supercoiling buffer [35 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 25 mM potassium glutamate, 2 mM spermidine, 2 mM ATP, 50 mg/L bovine serum albumin (BSA) and 90 mg/L yeast RNA in 5% (v/v) glycerol]. After 30 min, the reaction was stopped with 0.6% SDS. Drug-induced cleavage was performed in supercoiling

buffer with supercoiled pBR322 as substrate. The reactions were carried out at 30°C for 60 min in the presence of varied amounts of inhibitors and the gyrase–DNA complex was trapped by adding 0.2% SDS followed by proteinase K digestion (final concentration of 0.8 g/L) for 30 min. The reaction mixtures were resolved on a 0.8% agarose gel in 40 mM Tris–acetate buffer containing 1 mM EDTA. One or 10 U of enzyme was used for supercoiling and cleavage reactions, respectively. All experiments were performed in triplicate.

Definitions of potency

Based on their mechanism of cytotoxicity, the efficacy of the inhibitors was measured using the following parameters. The highest concentration of the inhibitor that failed to show any detectable inhibition of the supercoiling activity was termed the maximal non-effective concentration (MNEC), whereas the minimum concentration that produced complete inhibition was termed the IC_{100}. MNEC and IC_{100} were used to assess the efficacy of the ATPase inhibitors, since these provide information on both the lower and upper limits of the inhibition profile. Inhibitors that trap gyrase–DNA covalent complex were compared based on their CC_2 and maximum cleavage values. CC_2 was defined as the concentration of inhibitor required to stimulate basal cleavage by two-fold while maximum cleavage represents the fold increase in cleavage in the presence of saturating concentrations of the inhibitor.

Results and discussion

The supercoiling reaction catalysed by DNA gyrase consists of a series of steps. The enzyme binds to DNA and introduces a break in the DNA with concomitant formation of a protein–DNA covalent complex. This is followed by passage of another duplex through this gate and finally resealing of the break. Various inhibitors interfere with one or more of the substeps in the reaction.

Effect of ATPase inhibitors

The supercoiling reaction of DNA gyrase is driven by the energy derived from the hydrolysis of ATP. Therefore, monitoring the effect of ATPase inhibitors on the supercoiling activity is a reliable estimate of their potency. Experiments performed with *E. coli* gyrase have shown that cyclothialidines are more potent than coumarins.[10] Furthermore, they are more specific towards DNA gyrase than eukaryotic type II topoisomerases. However, their effect on mycobacterial gyrase has not as yet been assessed. As representatives of cyclothialidines, the compound Ro 09-1437 and its derivative Ro 48-2865 were tested for their ability to inhibit the enzyme activity (Figure 1). Both cyclothialidine analogues showed reduced activity against

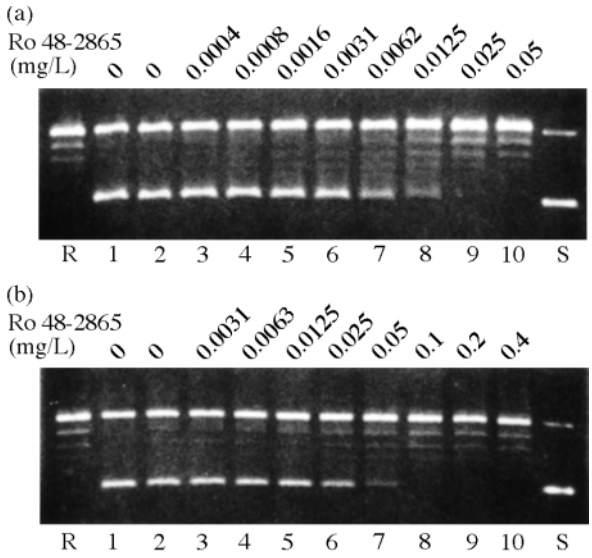

Figure 1. Supercoiling reactions were performed in the presence of various concentrations of cyclothialidine Ro 48-2865 (lanes 2–10) with (a) *E. coli* gyrase and (b) *M. smegmatis* gyrase. Lane 1 of both sets of supercoiling reactions were performed in the presence of 1% DMSO. S and R represent supercoiled and relaxed pUC18, respectively.

the *M. smegmatis* gyrase as compared with *E. coli* gyrase (Table 1). It is noteworthy that the *E. coli* enzyme was approximately eight-fold more susceptible to Ro 48-2865 than to the parent compound (Table 1).[36] In contrast, the mycobacterial gyrase did not exhibit any such differential susceptibility. On the other hand, novobiocin was able to inhibit mycobacterial gyrase at concentrations >0.0625 mg/L (Table 1). This was comparable to the efficacy with which the compound inhibits *E. coli* gyrase. The N-terminal portion of GyrB harbouring the ATPase activity is the maximally conserved region among different type II topoisomerases.[37] Therefore, it is not surprising that the ATPase inhibitors were able to inhibit both the enzymes. However, alterations in the primary sequence of DNA gyrase between different organisms appear to modulate the susceptibility of the enzyme to specific inhibitors.

Effect of ciprofloxacin, a fluoroquinolone

We tested the ability of representatives of the quinolones and fluoroquinolones to inhibit the supercoiling activity of DNA gyrase. Our results were comparable to published reports with *E. coli* and *M. smegmatis* gyrase.[25,38] As in the case of other bacteria, *M. smegmatis* gyrase was found to be more susceptible to fluoroquinolones than to quinolones. Although inhibitors like quinolones and their derivatives inhibit the overall DNA supercoiling activity of the enzyme, this inhibition and their cytotoxicity is a consequence of trapping the gyrase–DNA complexes.[11–14] This is exemplified in the case of CcdB and microcin B17, which do not inhibit the supercoiling reaction of DNA gyrase but trap the gyrase–DNA cleavage complex.[20,39] Therefore, the

Table 1. Inhibitory activities of novobiocin and cyclothialidines on the gyrases of *E. coli* and *M. smegmatis*

	MNEC (mg/L)		IC_{100} (mg/L)	
Inhibitor	*E. coli* gyrase	*M. smegmatis* gyrase	*E. coli* gyrase	*M. smegmatis* gyrase
Ro 09-1437	0.0125 ± 0.0011	0.025 ± 0.002	0.4 ± 0.02	0.4 ± 0.03
Ro 48-2865	0.00156 ± 0.00012	0.0125 ± 0.0008	0.05 ± 0.003	0.2 ± 0.02
Novobiocin	0.125 ± 0.006	0.0625 ± 0.0034	1.0 ± 0.012	0.5 ± 0.04

MNEC, maximal non-effective concentration of the inhibitor; IC_{100}, concentration of the inhibitor required for complete inhibition of supercoiling activity.
All values are mean ± S.D. of three independent experiments.

Table 2. Inhibitory activities of ciprofloxacin, etoposide and CcdB on the gyrases of *E. coli* and *M. smegmatis*

	CC_2 (μM)		Maximum cleavage	
Inhibitor	*E. coli* gyrase	*M. smegmatis* gyrase	*E. coli* gyrase	*M. smegmatis* gyrase
Ciprofloxacin	0.015 ± 0.001	0.17 ± 0.02	NS	NS
Etoposide	11.58 ± 0.62	34.59 ± 1.91	4.0 ± 0.3	13.3 ± 0.6
CcdB	0.483 ± 0.050	NDC	15.6 ± 1.1	NDC

CC_2, concentration of inhibitor required to stimulate cleavage two-fold; maximum cleavage, fold increase in cleavage in the presence of saturating concentration of inhibitor; NS, no saturation for the range of concentrations tested; NDC, no detectable cleavage.
All values are mean ± S.D. of three independent experiments.

extent of accumulation of the cleaved DNA in the presence of such inhibitors is a direct measure of the efficacy of the compound, and enables a comparison of all inhibitors that act by stabilizing the cleavage complex. Furthermore, the cleavage assay is a more sensitive assay than the supercoiling reaction.

When the effect of ciprofloxacin was tested on the supercoiling reaction, detectable inhibition (MIC) required 1.3 μM of the drug, in agreement with earlier reports.[38] In contrast, cleavage was doubled at concentrations (CC_2) as low as 0.17 μM (Figure 2 and Table 2). It should be noted that the *M. smegmatis* gyrase was less susceptible than *E. coli* gyrase in both supercoiling and cleavage assays. This was probably owing to the presence of several substitutions in the quinolone-resistance determining regions (QRDRs) of GyrA and GyrB of the *M. smegmatis* enzyme, including S83A in GyrA (amino acid position based on *E. coli*), and Y447R and S464N in GyrB.[40] In support of this view, mutations of these amino acids in *E. coli* have been shown to reduce the affinity of the enzyme for quinolones and fluoroquinolones.[2] The susceptibility of *M. smegmatis* DNA gyrase is comparable to the values obtained with *Staphylococcus aureus* topoisomerase IV.[41] It is noteworthy that in Gram-positive bacteria topoisomerase IV is the primary target for quinolones and their derivatives. However, in mycobacteria, DNA gyrase is the only type II topoisomerase identified so far. Furthermore, no homologue of topoisomerase IV has been identified in the *M. tuberculosis* and *Mycobacterium leprae* genomes, indicating that the sole target of the quinolone family of inhibitors in these organisms is DNA gyrase.

Effect of etoposide

Etoposide is a commonly prescribed antineoplastic drug.[42] It stabilizes the covalent complex of eukaryotic type II topoisomerases and DNA. Recently, it has been shown to trap the covalent intermediate of DNA and topoisomerase IV from *S. aureus*, albeit with a reduced efficiency compared with eukaryotic topoisomerase IIs.[41] In addition, both etoposide and ciprofloxacin appear to share a common binding site on topoisomerase IV, since ciprofloxacin-resistant mutants of topoisomerase IV show cross-resistance to etoposide.[41] The effect of etoposide on DNA gyrase from any source, including *E. coli*, has not as yet been assessed. Our results (Table 2) show the ability of etoposide to trap the gyrase–DNA complex. Both *E. coli* and *M. smegmatis* gyrase were susceptible to etoposide. Although *M. smegmatis* DNA gyrase had lower affinity for etoposide, it showed higher levels of cleavage under saturating concentrations of the drug. Therefore, in *M. smegmatis*, resistance to fluoroquinolones does not appear

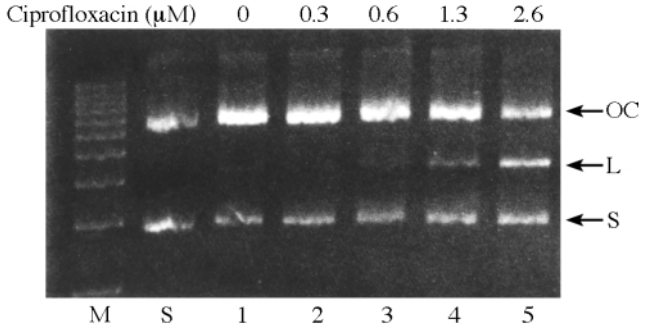

Figure 2. Cleavage reactions were carried out with *M. smegmatis* gyrase in the presence of various ciprofloxacin concentrations (lanes 1–5). S, supercoiled DNA; OC, open-circular DNA; L, linear pBR322 DNA. Lane M contains a 1 kb ladder (Life Technologies, NY, USA).

to translate directly to a decrease in susceptibility to etoposide. These results indicate that etoposide and fluoroquinolones are different in their interaction with DNA gyrase from *M. smegmatis* from that with *S. aureus*.

Effect of plasmid-borne proteinaceous inhibitors

Using purified *E. coli* DNA gyrase, CcdB has been demonstrated to trap the gyrase–DNA covalent complex *in vitro*.[18] Similar experiments with cell-free extracts showed an accumulation of the cleaved intermediate in the presence of microcin B17.[20] Cleavage reactions were carried out with both *E. coli* and *M. smegmatis* gyrase in the presence of different amounts of CcdB. With increasing amounts of CcdB, there was a concentration-dependent increase in the cleaved product with *E. coli* DNA gyrase (Figure 3a and Table 2). Under similar conditions, *M. smegmatis* gyrase was refractory to CcdB (Figure 3b). Comparison of the primary sequence of GyrA revealed that the amino acid residues in *E. coli* gyrase critical for CcdB action are not conserved in GyrA of *M. smegmatis* (G214E and R462Q), providing a possible molecular basis for the resistance. Similarly, *E. coli* DNA gyrase was susceptible to microcin B17 whereas the *M. smegmatis* enzyme was resistant (data not shown). Tryptophan at position 751, which is believed to render *E. coli* GyrB susceptible to microcin B17, is also present in the *M. smegmatis* gyrase, yet the latter is refractory to the peptide. These results indicate that there are additional residues involved in interactions with microcin B17, and the Trp751 is not the sole determinant of susceptibility to microcin B17.

In conclusion, one of the major findings in this study is the relative resistance of *M. smegmatis* DNA gyrase compared with *E. coli* gyrase. Only ciprofloxacin and etoposide show appreciable efficacy against the mycobacterial enzyme. Further modifications of these compounds would be needed to enhance their potency. The proteinaceous inhibitors CcdB and microcin B17 appear to be specific to

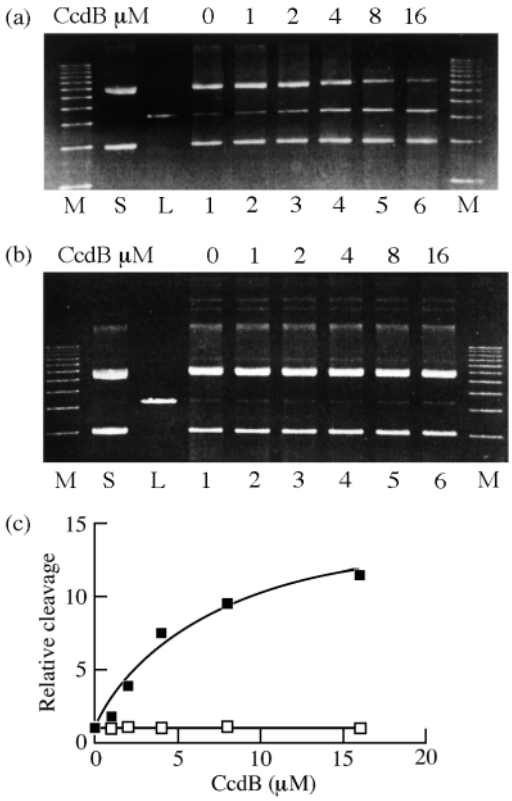

Figure 3. Cleavage reactions performed in the presence of different concentrations of CcdB with (a) *E. coli* gyrase and (b) *M. smegmatis* gyrase (lanes 1–6). S and L represent supercoiled and linear pBR322 DNA, respectively. Lane M contains a 1 kb ladder (Life Technologies). (c) Graphical representation of the results (■, *E. coli*; □, *M. smegmatis*). Relative cleavage is the amount of cleavage product seen in the presence of CcdB normalized to the intrinsic cleavage produced by the enzyme alone.

E. coli gyrase. The lack of inhibition of *M. smegmatis* gyrase by these molecules correlates well with the observation that the genes encoding these proteins are present on plasmids specific to Gram-negative bacteria. Thus, it would not be surprising if these inhibitors have evolved high specificity to the gyrases they primarily encounter, and is consistent with the 'selfish' behaviour of plasmids.[16,43]

Conclusion

Our study constitutes a detailed analysis of the effect of various groups of inhibitors on the DNA gyrase from a Gram-positive bacteria. Such an extensive study has previously only been performed with the enzyme from *E. coli*. This analysis indicates subtle differences in the enzyme structure between the two very divergent species. Moreover, since gyrase has already been used as a molecular target for anti-mycobacterial therapy, the present investigation also gives direction to the development of modified compounds as better therapeutic agents.

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