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SURVEY AND SUMMARY

Bacterial topoisomerase I as a target for discovery of antibacterial compounds

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ABSTRACT

Bacterial topoisomerase I is a potential target for discovery of new antibacterial compounds. Mutant topoisomerases identified by SOS induction screening demonstrated that accumulation of the DNA cleavage complex formed by type IA topoisomerases is bactericidal. Characterization of these mutants of *Yersinia pestis* and *Escherichia coli* topoisomerase I showed that DNA religation can be inhibited while maintaining DNA cleavage activity by decreasing the binding affinity of Mg(II) ions. This can be accomplished either by mutation of the TOPRIM motif involved directly in Mg(II) binding or by altering the charge distribution of the active site region. Besides being used to elucidate the key elements for the control of the cleavage-religation equilibrium, the SOS-inducing mutants of *Y. pestis* and *E. coli* topoisomerase I have also been utilized as models to study the cellular response following the accumulation of bacterial topoisomerase I cleavage complex. Bacterial topoisomerase I is required for preventing hypernegative supercoiling of DNA during transcription. It plays an important role in transcription of stress genes during bacterial stress response. Topoisomerase I targeting poisons may be particularly effective when the bacterial pathogen is responding to host defense, or in the presence of other antibiotics that induce the bacterial stress response.

INTRODUCTION

Topoisomerases catalyze the interconversion of DNA topological isomers via coupling of DNA phosphodiester bond cleavage and religation with the passage of DNA through the break. By maintaining global DNA supercoiling at optimal level and removing local topological barriers, DNA topoisomerases play vital roles in DNA replication, transcription, repair and recombination (1). Topoisomerases are divided into different subfamilies based on their mechanisms and sequence similarities (2–4). Type I topoisomerases cleave and rejoin one strand of DNA while type II topoisomerases cleave and rejoin a double strand of DNA during catalysis. Human topo IB, IIA and bacterial topo IIA enzymes are well utilized clinical targets for anticancer and antibacterial chemotherapy (5–9). These topoisomerase targeting compounds initiate the cell killing process by either stabilizing or increasing the accumulation of the covalent complex formed between the enzyme and cleaved DNA and are called 'topoisomerase poisons' (9–11). Compounds that interact with type IA topoisomerases with high specificity to increase the level of the covalent complex remain to be identified. The emergence of bacterial pathogens resistant to multiple antibacterial drugs in both the hospital and community setting is a serious global public health problem, presenting an urgent need for discovery of new classes of antibacterial compounds. Based on the similarities in the topoisomerase mechanisms, it should be productive to identify small molecules that can act as poisons of bacterial type IA topoisomerases.

TYPE IA TOPOISOMERASES BACTERIA

There is at least one type IA topoisomerase found in each bacterial genome (12). Topo I is present in all bacteria and is the major activity responsible for removal of excess negative supercoiling (13). In *Escherichia coli*, the promoters of topo I and gyrase genes are under homeostatic control by DNA supercoiling to maintain the global level of supercoiling (14,15). Topo III is found in only some of the bacterial genomes. *In vitro* it is much more efficient in catalyzing DNA decatenation reaction than relaxation (13). *Escherichia coli* topo III has been proposed to play a role in resolving RecQ associated recombination intermediates (16). Transposon insertion or deletion mutants in the *topA* gene coding for topo I could be isolated from

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E. coli, Salmonella typhimurium and *Shigella flexneri* (17–20). Attempts to isolate transposon insertion mutants in the *topA* gene were unsuccessful in *Mycobacterium tuberculosis* and *Helicobacter pylori* (21,22) suggesting that topo I might be essential in these bacteria. This should be further investigated by additional genetic studies. In *E. coli*, topo III is also not essential but absence of both type IA topoisomerases resulted in chromosomal segregation defect (23). It was proposed that a type IA topoisomerase activity is required in general for resolving recombination intermediates involving single strand DNA passage (1). A broad spectrum type IA topoisomerase poison that can act on both topo I and topo III would have a target always present in any bacteria.

Even though *E. coli topA* mutants are viable, growth at low temperature requires topo I function (24,25). During transcription, the movement of the RNA polymerase complex generates positive supercoils in the DNA template ahead of it and negative supercoils behind it (26). Topo I activity is needed for removal of the negative supercoils to prevent hypernegative supercoiling and R-loop formation (27,28). The importance of topo I function in transcription is also illustrated by the direct protein–protein interaction between *E. coli* topo I and RNA polymerase (29,30).

FUNCTION OF BACTERIAL TOPOISOMERASE I IN STRESS RESPONSE AND PATHOGENESIS

The role of topo I in relaxation of transcription-induced negative supercoiling is probably especially important during stress response when a large number of stress genes have to be induced rapidly for survival (31). Transcription of *E. coli topA* gene is under control of multiple promoters recognized by σ32, σ38 in addition to σ70 (32,33). Besides these alternative σ factors, *topA* transcription is also regulated by binding of Fis to the promoter region (34). Topo I function and regulation have been shown to be important for *E. coli* response to high temperature and oxidative stress (35–38). RNase H overproduction can partially restore the σ32–dependent stress genes transcription defect in the absence of *topA*, indicating that R-loop formation from hypernegative supercoiling at heat shock genes loci is responsible for the effect of the *topA* mutation. The response to high temperature and oxidative stress is an important element of bacterial pathogen adaptation against host defense. In *H. pylori*, the *topA* gene has been shown to be up-regulated by prolonged acid exposure (39). Loss of topo I function in *E. coli* affects transcription of the acid resistance genes *gadA* and *gadBC* involving a mechanism independent of R-loop suppression (40). It was shown recently that a pathway of σ38 stress response involves unwrapping of the poised inactive transcription complex by DNA relaxation to allow activation (41). The relaxing activity of topo I may play a role via this mechanism in the activation of some of the promoters that are stimulated by DNA relaxation (42,43). The important role of topo I in bacterial stress response and pathogenesis implies that if topo I activity is reduced as a defensive adaptation for an antibiotic acting as type IA topoisomerase poison, the ability of the pathogen to survive in the host environment may be compromised.

Bacterial genes related to pathogenesis and virulence have been shown to be sensitive to *topA* mutation. These include the *fooB* gene for fimbriae $F165_1$ in pathogenic *E. coli* 4787 (44) and the thermally regulated invasive genes of *S. flexneri* (20). The *invA* gene of *S. typhimurium* was poorly expressed in a *topA* mutant, and this correlated with the ability of *S. typhimurium* to penetrate tissue culture cells (45). Signature-tagged transposon mutagenesis has identified *topA* to be one of the genes affecting survival of *Yersinia enterocolitica* in animal host (46). In addition, *topA* gene was among those found to be highly expressed by avian pathogenic *E. coli* (APEC) in infected tissues (47).

The lethal mechanism of many bactericidal antibiotics based on different mechanisms of action, including quinolones and ampicillin, have been shown to involve at least in part the formation of reactive oxygen species (48–50). Since bacteria in general would respond to such antibacterial antibiotics with transcription of stress regulons, topo I is expected to interact extensively with negatively supercoiled DNA at the induced transcription loci, and provide additional opportunity for the topo I cleavage complex to be trapped by an inhibitor. Topo I inhibitors that act as poisons may therefore be particularly effective in combination therapy with other antibiotics.

CELL KILLING BY BACTERIAL TOPOISOMERASE I MUTANTS THAT ACCUMULATE THE COVALENT CLEAVAGE COMPLEX

Since molecules that can act effectively as bacterial topo I poisons have not been previously identified, it is important to demonstrate that trapping of topo I cleavage complexes on single-stranded DNA will indeed lead to bacterial cell death, just as tapping of type IIA topoisomerases on double-stranded DNA would. This would validate the targeting of bacterial topo I in the search for novel antibacterial compounds. It is known from previous work that the SOS response of *E. coli* is induced by the trapping of gyrase cleavage complex by quinolones (51). Topo I mutations that mimic the action of a topoisomerase poison and result in increased accumulation of the cleavage complex may be expected to also induce the SOS response. The isolation of such topo I mutants was achieved by screening for SOS-inducing recombinant mutant *Yersinia pestis* topo I expressed in *E. coli* under the control of the tightly-regulated BAD promoter (52). A pool of mutagenized plasmid pYTOP expressing random mutants of *Y. pestis* topo I was first isolated in the presence of 2% glucose to suppress the expression of any potentially lethal mutant that accumulate the cleavage complex. The mutagenized plasmid was then transformed into *E. coli* JD5 strain with *dinD1*::*lacZ* fusion. Induction of the DNA damage SOS response would result in synthesis of β-galactosidase from activation of the *dinD*1 promoter and formation of blue colonies on Xgal plate. SOS-inducing *Y. pestis* topo I mutants were identified in such blue colonies in the presence of low concentration of arabinose

to activate the BAD promoter. Induction of an SOS-inducing mutant topo I with high concentration of arabinose resulted in ~4 logs loss of viable counts after 2 h (52). The mutation responsible for this phenotype was identified to be a Gly to Ser substitution in the TOPRIM motif DxDxxG conserved in nucleotidyl transferases (53). Mutant topoisomerase with this Gly to Ser substitution was found to be defective in DNA rejoining after formation of the covalent DNA cleavage complex (52). This result demonstrated that accumulation of the covalent cleavage complex of bacterial topo I could indeed lead to rapid bacterial cell death and validated bacterial type IA topoisomerases as useful targets for discovery of novel bactericidal compounds. The Gly to Ser mutation was the only substitution found at that position to have the SOS-inducing and cell killing phenotypes for *Y. pestis* topo I. The other more bulky substitutions eliminated DNA cleavage activity. A Met to Val substitution immediately following the active site tyrosine was present in the original SOS inducing mutant (52) and was found to enhance DNA cleavage without inhibiting DNA religation (54).

Mg^{2+} ions are required for DNA rejoining and DNA relaxation by type IA topoisomerases (13,55). The aspartates in the TOPRIM motif DxDxxG in *E. coli* topo I have been shown to coordinate two Mg^{2+} ions (56). Mg^{2+} binding by the TOPRIM motif is found to be critical for the DNA cleavage-religation equilibrium of bacterial topo I. Characterization of the SOS-inducing and cell killing topo I mutants demonstrated that there are at least three mechanisms of decreasing Mg^{2+} binding affinity and inhibiting DNA religation (Figure 1). The Gly to Ser mutation at the TOPRIM motif probably distorted the structure of the TOPRIM domain as the first mechanism of perturbing DNA cleavage-religation. Replacement of the conserved Met adjacent to the active site tyrosine with Arg also resulted in reduced Mg^{2+} binding, inhibition of DNA religation and the cell killing phenotype. Molecular modeling was in agreement with increased positive charge in the active site region due to the substitution of the neutral Met with the positively charged Arg (57). The change in electrostatic potential in the active site provided a second mechanism of perturbing Mg^{2+} binding and DNA cleavage-religation. It is also expected that interfering with the metal-ligand interaction directly would provide a third mechanism of perturbing Mg^{2+} binding and DNA cleavage-religation. Results from our recent experiments showed that an Asn substitution at the first TOPRIM motif Asp residue gave rise to an extremely toxic topo I mutant, and the Asn substitution at the second TOPRIM motif Asp residue also resulted in a mutant that was lethal when induced (B. Cheng *et al.*, manuscript in preparation).

While the involvement of Mg^{2+} ions is widely found in many cellular catalytic mechanisms, there is precedence for small molecules affecting Mg^{2+} interactions being identified as specific inhibitors of a cellular process which have been approved in human therapy. HIV-1 integrase is an important new target for anti-viral therapy for cases resistant to existing drugs against HIV-1 reverse transcrptase and protease (58,59). The integrase mechanism is similar to the type IA topoisomerase mechanism in the use of two aspartates and one glutamate (56)

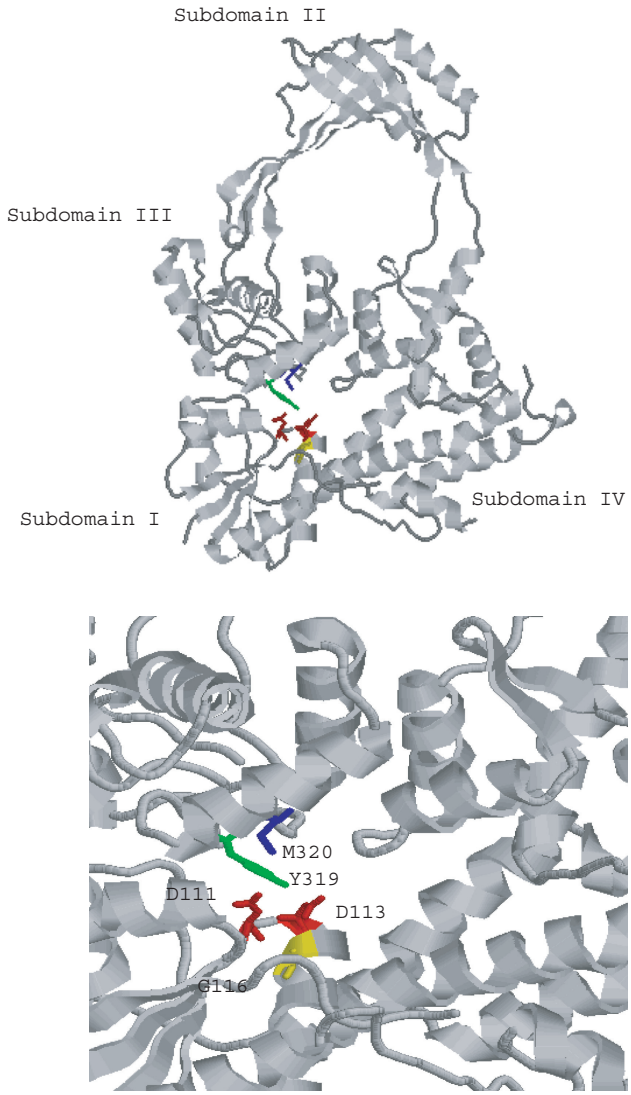

Figure 1. Structure of the 67 kDa N-terminal fragment of *E. coli* topo I (PDB ECL1) and the close-up view of the active site showing the nucleophilic tyrosine Y319 and the residues in the TOPRIM motif and active site region where mutations can result in cell killing due to inhibition of Mg^{2+} binding and DNA religation.

to coordinate two divalent ions and bring about protein conformational change (60–62). Small molecules that can chelate the divalent ions in the HIV-1 integrase active site have been identified and one, raltegravir has been approved for treatment of AIDS patients (63,64). Therefore it is not unreasonable to expect that it may be possible to inhibit the interaction between bacterial topo I and metal ions in the active site with a small molecule and achieve accumulation of the DNA cleavage complex for antibacterial therapy.

While the TOPRIM motif and surrounding residues around the active site tyrosine (Figure 1) have been identified to be important for controlling DNA cleavage/religation, other regions of the bacterial topo I protein could also affect the level of accumulation of the covalent cleavage complex. Subdomain III with the 5′phosphate of the cleaved DNA bound covalently to the active site tyrosine

needs to separate from subdomains I and IV as the enzyme transitioned from the closed structure (Figure 1) to a more open structure during the catalytic cycle for DNA strand passage to take place. Subdomain I then must move back to near its original position before DNA religation can occur. Mutations or small molecules that can stabilize the intermediate complex with subdomain III separated from subdomains I and IV by affecting the protein conformational changes could also lead to increased accumulation of the cleavage complex in vivo and result in cell killing. It is hopeful that further characterization of the SOS-inducing topo I mutants would identify regions in the topo I structure that may be potential binding sites for such small molecules.

CELLULAR RESPONSE TO TOPOISOMERASE I CLEAVAGE COMPLEX AND THE CELL DEATH PATHWAY

The bacterial topo I mutants that accumulate the cleavage complexes were utilized to study the cellular response to topo I cleavage complex and the repair pathway in *E. coli*. The homologous recombination function of RecA was found to be required for repair of topo I cleavage complex. Induction of the SOS response by the recombination deficient RecA718 protein was found to be insufficient for repair of topo I mediated DNA cleavage (65) Quinolones are known to induce the SOS response of *E. coli* via the RecBCD pathway (66). Double-strand DNA breaks and chromosomal fragmentation occur after trapping of the cleavage complex between the gyrase A subunits and both strands of DNA (67,68). Although topo I cleaves only a single-strand of DNA at a time, RecBCD function was also found to be required for induction of the SOS response by RecA (65). The *E. coli* RecBCD complex unwinds and degrades DNA at double strand breaks with free DNA ends until the RecBCD activity is modified by encountering a Chi site on DNA. The Chi-modified RecBCD nuclease activity generates a 3′ single-stranded overhang and facilitates the assembly of a RecA filament on this single-stranded DNA region downstream of the Chi site (69). Mutations in the single-strand gap repair pathway genes *recF* and *recR* were found to have no effect on SOS induction or viable colony counts following accumulation of the topo I cleavage complex (65). It remains to be determined how the single strand break covalently linked to topo I protein is converted to a double strand break with free ends that can be processed by the RecBCD complex.

The quinolone induced gyrase cleavage complex blocks DNA replication, but ongoing DNA replication is not necessary or sufficient for the lethal effect of the quinolones (11,70). Depending on the structure of the quinolone, inhibition of protein synthesis following quinolone addition by chloramphenicol may or may not offer protection. This suggests that there is more than one pathway involved for the lethal action of quinolones (11). It is possible that a newly synthesized protein may be required for one of the cell killing pathways. In eukaryotes, Tyr-DNA phosphodiesterase (Tdp1) activity has been shown to cleave the linkage between 3′ DNA phosphate and type IB topoisomerases (71,72), and may in addition also be involved in the processing of the type IIA topoisomerases linked to the 5′ DNA phosphate (73,74). Homologues of Tdp1 have not been identified in bacterial genomes so it is not known if a bacterial Tyr-DNA phosphodiesterase is available to cleave the linkage between the type IA and type IIA topoisomerase proteins and the 5′ phosphates of the cleaved bacterial DNA.

INHIBITORS OF BACTERIAL TOPOISOEMRASE I

Even though DNA gyrase is the primary target of quinolones in *E. coli*, certain quinolones including perfloxacin, ciprofloxacin, norfloxacin and ofloxacin have been shown to inhibit the relaxation activity of *E. coli* topo I at concentrations 10-fold or higher of that required to inhibit the supercoiling activity of *E. coli* gyrase (75,76). Nevertheless, *E. coli* topo I was found to be significantly more sensitive to these quinolones than calf thymus topoisomerase I and II.

Inhibition of *E. coli* topo I by phospholipids has also been reported (77). The inhibition of the *in vitro* relaxation activity by cardiolipin (Figure 2A) could be suppressed by chlorpromazine. Treatment of *E. coli* cells with chlorpromazine resulted in relaxation of plasmid DNA that was dependent on the function of the *topA* gene. This suggested that chlorpromazine may interfere with the *in vivo* interaction between *E. coli* topo I and phospholipids in *E. coli* cells (77).

Escherichia coli Tn5 transposase protein has been shown to copurify with *E. coli* topo I and inhibit its relaxation activity (78). The titration of topo I activity by overexpressed Tn5 transposase leads to filamentation, aberrant nucleoid segregation and cell death (79). The lethality from overexpression of Tn5 transposase could be suppressed by chromosomal mutations that increased the level of topo I protein (80). The N-terminal amino acids of Tn5 transposase are required for the interaction with topo I and the resulting lethal effect from Tn5 transposase overexpression (78,79).

In order to identify small molecules as leads for bacterial topo I poisons, a cell based high-throughput assay was developed utilizing *E. coli* cells with enhanced permeability to small molecules, and overexpressing *Y. pestis* topo I with the *dinD1::luxCADBE* luciferase fusion as reporter (81). The goal was to identify small molecules that could induce higher level of SOS response when wild-type recombinant topo I was overexpressed as compared to the recombinant topo I with the active site nucleophile tyrosine residue substituted with alanine. Three small molecules (Figure 2B) capable of enhancing bacterial topo I DNA cleavage and inhibiting the relaxation activity have been identified (81). These compounds had antibacterial activity against the gram positive *Bacillus subtilis* but not *E. coli* cells with normal permeability, and may not have the desired specificity against bacterial topo I (81). Compound 1 is the natural product stephenanthrine, a phenanthrene alkaloid. It has structural similarities to benzo[c]phenanthridines and protoberberine alkaloids

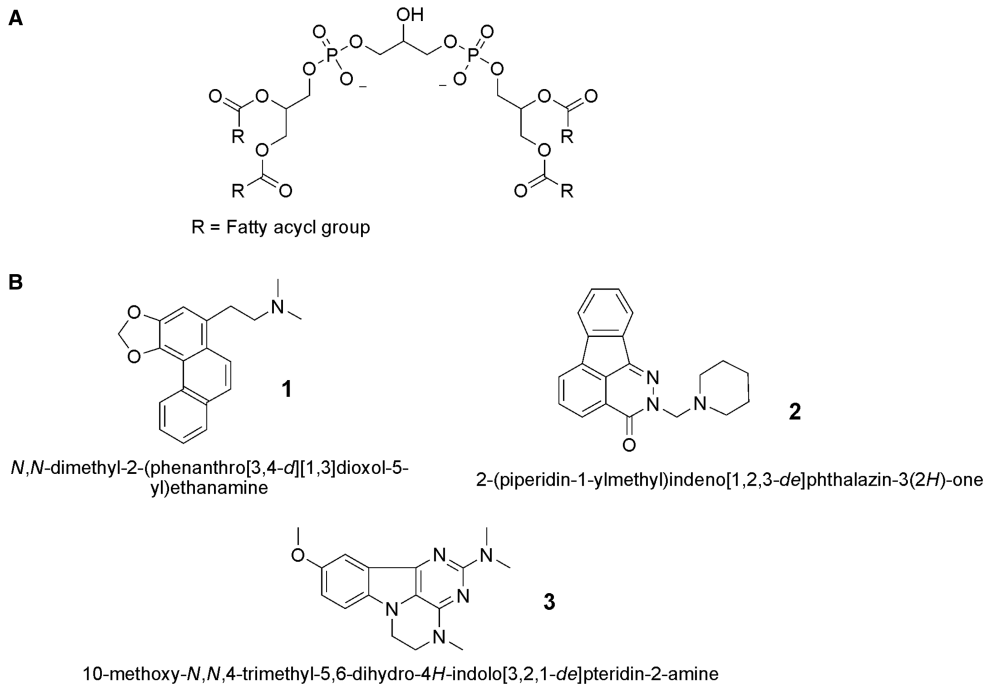

Figure 2. Structures of compounds shown to inhibit relaxation activity of *E. coli* topo I. (**A**) cardiolipin (77). (**B**) Small molecule leads identified by high-throughput screening (81).

that have been shown to enhance the DNA cleavage of mammalian topoisomerase I and II (82). Minor groove binders including the bisbenzimide Hoechst 33342 are known to exhibit anti-tumor activity due to inhibition of human topoisomerase I and trapping of the human topoisomerase I cleavage complex (83). More recently, modification of Hoechst 33342 has provided novel ligands that could clear bacterial infections from mammalian cell culture without apparent cytotoxicity to the mammalian cells, and these ligands preferentially inhibited *E. coli* topo I over human topo I *in vitro* (Vibha Tandon, personal communications). These results suggest that modification of ligand structures can potentially shift their specificity from inhibiting mammalian topoisomerase I towards targeting bacterial topo I, improving their antimicrobial potential while limiting the cytotoxicity.

FUTURE DIRECTIONS

Additional screenings should be carried out to identify compounds that can act as topo I poisons so that they can be developed into leads for new antibacterial therapy. Two high-throughput assays have been developed assaying DNA supercoiling by DNA gyrase and DNA relaxation by eukaryotic topo I and II as well as *E. coli* topo IV (84). These assays should be applicable to identifying small molecules that can inhibit relaxation by bacterial topo I. Some of the relaxation inhibitors may act by inhibiting DNA religation and be useful as a bacterial topo I poison. Other *in vitro* high-throughput assays that can directly measure the level of the topo I cleavage complex would be extremely useful for identifying new leads.

With sites in bacterial topo I structure important for the control of the DNA cleavage-religation equilibrium being located by the SOS-inducing mutations, it should be possible to identify small molecules that can interact with these sites in the enzyme. The virtual screening approach has been used to discover novel gyrase inhibitors (85), and should be attempted for identifying leads for discovery of bacterial topo I poisons.

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