ABSTRACT

Bacterial 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 pathogenresistant 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.

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).
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 patho-

As a result of 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 fimbrae F1651 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 quino-

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::λacZ fusion. Induction of the DNA damage SOS response would result in synthesis of β-galactosidase from activation of the dinD1 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 inte-rase is an important new target for anti-viral therapy for cases resistant to existing drugs against HIV-1 reverse transcriptase 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) 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' phosphatase 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’ DNA phosphate and 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 TOPOISOMERASE 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 stephanthrine, a phenanthrene alkaloid. It has structural similarities to benzo[cd]phenanthridines and protoberberine alkaloids.
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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