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Assessing Sensitivity to Antibacterial Topoisomerase II Inhibitors

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Abstract

Both prokaryotes and eukaryotes have two major classes of topoisomerases that make transient single- or double-strand cuts in DNA. While these enzymes play critical roles in cellular processes, they are also important targets of therapeutic agents. This unit describes assays to use in characterizing topoisomerase II-targeting agents in vitro and in bacterial cells. It provides protocols for characterizing the action of small molecules against bacterial type II topoisomerases in vitro and the in vivo effects of putative topoisomerase II-targeting antibiotics, as well as for measuring trapped enzyme/DNA covalent complexes, the major cytotoxic lesion induced by fluoroquinolones.

Keywords

topoisomerase; gyrase; antibiotic; topoisomerase poison; fluoroquinolone; MIC; MPC; supercoil

Introduction

Topoisomerases play essential roles in DNA metabolism, and they are important targets of therapeutic agents (e.g., anticancer drugs, such as ectoposides, and antibiotics). Both prokaryotes and eukaryotes have two major classes of topoisomerases: type I enzymes make transient single-strand cuts in DNA, whereas type II enzymes generate transient DNA double-strand breaks during the enzyme reaction cycle (see Tables 3.13.1 and 3.13.2). Although eukaryotic type I enzymes can be targets of drugs, this unit describes assays for characterizing topoisomerase II-targeting agents in vitro and in vivo in bacterial cells.

The first type II topoisomerase identified was the bacterial enzyme DNA gyrase. (See Table 3.13.1 for the complement of topoisomerases in *E. coli*.) This enzyme catalyzes a unique ATP-dependent introduction of negative supercoils into DNA (see legend to Figure 3.13.1). Discovery of the two subunits of DNA gyrase was accomplished in part through the identification of the genes encoding resistance to nalidixic acid and novobiocin (Gellert et al., 1976, Sugino et al., 1977). These antibacterial agents target the GyrA and GyrB subunits of gyrase. Many bacteria contain a second type II topoisomerase, called topoisomerase IV because it was the fourth topoisomerase identified in *E. coli* (Kato et al., 1990). This enzyme relaxes negative and positive DNA supercoils, (Adams et al., 1992, Zechiedrich and Cozzarelli, 1995), although it does not introduce negative supercoils (Kato et al., 1990, Peng and Mariani, 1993). Figure 3.13.1 shows a diagram of key topoisomerase II reactions.

Prokaryotic type II topoisomerases are the targets of fluoroquinolones (Fig. 3.13.2), the most widely prescribed antibiotics in the United States (Linder et al., 2005). Fluoroquinolones are

“topoisomerase poisons” that trap a covalent enzyme-DNA complex, resulting in DNA damage that includes DNA double-strand breaks and protein covalently attached to DNA. The ability to generate enzyme-mediated DNA damage is intrinsic to the efficacy of fluoroquinolones as antibacterial agents (see Background Information). Therefore, unique assays measuring the ability of topoisomerases to cleave DNA, as well as more standard assays for measuring inhibition of enzyme activity, are important in characterizing topoisomerase-targeting agents.

Basic Protocols 1, 2, and 3 and an Alternate Protocol are used to characterize the action of small molecules against bacterial type II topoisomerases in vitro. Basic Protocols 1 and 2 describe assays for measuring inhibition of enzyme activity. One of the bacterial type II topoisomerases (topoisomerase IV) has a major role in unlinking catenated (circular, linked) replicated chromosomes (see Fig. 3.13.1). It is also able to relax DNA supercoils and is an important target for new chemical entities (NCEs) that target type II topoisomerases. The other bacterial type II topoisomerase (DNA gyrase) has the unique ability to introduce negative supercoils in DNA. In general, DNA gyrases are rather weak DNA decatenases (Ullsperger and Cozzarelli, 1996; Zechiedrich et al., 1997; Stone et al., 2003). Basic Protocol 1 can be employed to measure in vitro inhibition of decatenation by DNA gyrases, even though inhibition of DNA supercoiling is the more physiologically relevant assay (see Basic Protocol 2) because this is the enzyme's primary cellular role (Zechiedrich et al., 2000). Basic Protocol 3 and the Alternate Protocol describe assays for detecting trapped enzyme/DNA covalent complexes, the major cytotoxic lesion induced by fluoroquinolones.

NCE effects must also be assessed in vivo, and a second set of protocols (Basic Protocols 4, 5, and 6) is used to characterize the in vivo effects of putative topoisomerase II-targeting antibiotics. Assays are described for measuring the efficacy of new agents in inhibiting the growth of *E. coli* strains bearing mutations in the genes encoding either DNA gyrase or DNA topoisomerase IV that confer fluoroquinolone resistance. These assays are predicated on the assumption that a useful property of a new topoisomerase-targeting drug may be substantial growth inhibitory activity against bacterial strains resistant to traditional fluoroquinolones.

With all antibiotics there is a possibility of selecting for resistant mutants at concentrations above the minimum inhibitory concentration (MIC; see Basic Protocol 4). If partial resistance can easily arise, some colonies will grow at drug concentrations above the MIC. The mutant prevention concentration (MPC; see Basic Protocol 5) is the concentration of drug that completely blocks the growth of all colonies. If the MIC is close to the MPC, then resistance to low levels of the drug occurs infrequently. Conversely, an MPC much greater than the MIC suggests the agent is more likely to select for low levels of resistance.

In bacterial cells, the DNA is maintained in a negatively supercoiled state, which requires DNA gyrase activity. Inhibition of gyrase leads to reduced levels of DNA supercoiling, resulting in a relaxed state. While DNA supercoiling of bacterial chromosomal DNA can be measured (Sinden et al., 1980; Sinden and Pettijohn, 1981), such measurements are technically demanding. A simpler approach is to use an autonomously replicating plasmid as a surrogate for the supercoiling of chromosomal DNA (see Basic Protocol 6).

Basic Protocol 1: Determination of Inhibition of Bacterial Type II Topoisomerases: Assays for Inhibition of DNA Gyrase and Topoisomerase IV

Type II topoisomerases are unique in their ability to catalyze the decatenation of intact double-stranded DNA, used in vivo to separate replicated DNA molecules prior to cell

division (Nitiss, 1998; Wang, 2002). The assay described below utilizes the kinetoplast DNA from *Crithidia fasciculata*, which is a large network of interlocked (catenated) circles (Marini et al., 1980). The catenated circles are unable to enter an agarose gel. Type II topoisomerases decatenate the circles from the network, and upon decatenation, the freed circles are detected as a discrete band on an agarose gel. Both type I and type II topoisomerases catalyze relaxation of supercoiled DNA, but because type I topoisomerases cannot catalyze decatenation, the assay is specific for type II topoisomerases. This assay is the simplest method for determining whether an NCE is a gyrase and topoisomerase IV inhibitor, although it is not necessarily relevant to the mode of action for in vivo inhibition of type II topoisomerases. A similar assay in *UNIT 3.3* allows determination of inhibitory activity by an experimental agent towards human topoisomerase II enzymes.

CAUTION: Ethidium bromide is a mutagen. Wear gloves when handling, and properly dispose of all solutions.

Materials

- 10× topoisomerase IV reaction buffer (see recipe; also used for gyrase)
 - 20 mM ATP (diluted with H₂O from purchased stock solution; store in small aliquots up to 6 months at −20°C)
 - Kinetoplast DNA solution (TopoGen)
 - New chemical entity (NCE) to be tested
 - Positive control (e.g., ciprofloxacin; Sigma-Aldrich), optional
 - Purified topoisomerase IV and/or DNA gyrase (TopoGen or Inspiralis; see Critical Parameters)
 - 250 mM EDTA
 - 5× loading dye (see recipe)
 - 0.8% agarose gel (see Voytas, 2000) prepared in 1× TAE (see recipe for 50× TAE buffer)
 - 10 mg/ml ethidium bromide stock solution: store at room temperature, protected from light
 - Camera suitable for gel documentation
 - Additional reagents and equipment for performing agarose gel electrophoresis, ethidium bromide staining, and gel photography (Voytas, 2000)
1. For each experimental reaction add to a 1.5-ml microcentrifuge tube:
 - 2 µl of 10× topoisomerase IV reaction buffer
 - 1 µl of 20 mM ATP
 - 200 ng kinetoplast DNA (e.g., 1 µl of a 200 µg/ml stock).
 Include a tube for a control reaction (no enzyme, no inhibitor) and a positive control (if desired).
 2. Adjust volumes with distilled water so that the final reaction volume in each tube (including that of the enzyme and NCE added in steps 3 and 4) will be 20 µl.

This protocol is described as if one reaction is being conducted. When performing more than five reactions it is useful to prepare a master mix

that contains all of the components common to all reactions. For example, if conducting nine reactions, prepare sufficient master mix for ten reactions (to allow for loss and pipetting errors) that contains 20 μ l of 10 $\times$ topoisomerase IV reaction buffer, 10 μ l of 20 mM ATP solution, and 10 μ l of kinetoplast DNA. Then dispense 4 μ l into each of nine tubes, add the appropriate volume of water, inhibitor, and enzyme. Remember to allow for control reactions that include no enzyme, no inhibitor and, if used, a positive control. This strategy is applicable to Basic Protocols 1 to 3 and the Alternate Protocol. It minimizes pipetting errors.

3. Add varying amounts of NCE or positive control to the topoisomerase reaction tubes.

Appropriate starting concentrations of NCE are from 0.5 to 100 μ M.

If DMSO is used to dissolve the NCE, the final concentration of the solvent should not exceed 0.5%. This may limit the maximum concentration of an NCE that can be tested.

4. Add purified topoisomerase IV or gyrase protein to the tubes.

For topoisomerase IV, an appropriate concentration of enzyme is ~50 fM of tetramer. DNA gyrase catalyzes decatenation much less efficiently than topoisomerase IV. Therefore, inhibition of supercoiling (see Basic Protocol 2) is the preferred assay. If inhibition of decatenation by DNA gyrase is to be measured, a starting concentration of 300 to 1000 fM (0.2 to 1 μ g) is recommended.

5. Incubate 30 min at 37°C. Terminate the reaction by adding 1.5 μ l of 250 mM EDTA.
6. Add 5 μ l of 5 $\times$ loading dye to each tube and load the contents on a 0.8% agarose gel prepared in 1 $\times$ TAE. Electrophorese 2 to 3 hr at 5 to 10 V/cm (see Voytas, 2000).
7. Stain the gel 30 min in 1 μ g/ml ethidium bromide staining solution (diluted from 10 mg/ml stock), followed by destaining in water for 5 to 10 min.

CAUTION: Ethidium bromide is a mutagen. Wear gloves when handling, and properly dispose of all solutions containing ethidium bromide.

8. Visualize the gel lanes with a UV transilluminator and photograph (see Voytas, 2000).
9. Determine the inhibitor-to-topoisomerase ratio needed to fully block decatenation of kinetoplast DNA under standard conditions.

An example of a decatenation assay with topoisomerase TV in the presence of ciprofloxacin is shown in Figure 3.13.3. While analysis by densitometry could be applied, it is not typically used for this type of experiment.

Basic Protocol 2: In Vitro Inhibition of Supercoiling Activity of Gyrase

This assay (adapted from Gellert et al., 1976) measures the ability of an NCE to inhibit the supercoiling activity of gyrase in vitro. Under normal reaction conditions gyrases induce ATP-dependent supercoiling in circular DNA substrates. If an agent inhibits gyrase activity, the enzyme will no longer induce supercoiling. Thus, the level of supercoiling of a relaxed plasmid substrate (measured by its movement through an agarose gel) will remain

unchanged. This assay does not directly demonstrate *how* gyrase is inhibited. For example, fluoroquinolones (which can be cellular poisons; see Commentary, Background Information) and coumarins (which competitively inhibit ATP binding to a type II topoisomerase) will both inhibit the supercoiling activity of the enzyme (see Anticipated Results), despite their very different mechanisms of inhibition. This assay measures both types of inhibition, that of fluoroquinolones as well as agents that do not interfere with the breakage-reunion reaction (e.g., NCEs that inhibit the supercoiling activity of gyrase by competitively inhibiting ATP binding; see Commentary, Anticipated Results).

CAUTION: Phenol is caustic and can cause severe burns. Solutions containing phenol should be disposed of with compatible organic waste.

CAUTION: Ethidium bromide is a mutagen. Wear gloves when handling, and properly dispose of all solutions.

Materials

0.4 µg/ml DNA substrate: relaxed, covalently closed plasmid DNA (e.g., pBR322; New England Biolabs or see Critical Parameters)

Eukaryotic topoisomerase I (TopoGen)

5× gyrase reaction buffer (see recipe)

New chemical entity (NCE) to be tested

Gyrase

1 mM ciprofloxacin (Sigma-Aldrich) or other topoisomerase II inhibitor as a positive control

Purified gyrase (TopoGen or Inspiralis; see Critical Parameters)

Gyrase dilution buffer (see recipe)

28 mM ATP (diluted with H₂O from purchased stock solution; store up to 6 months in small aliquots at −20°C)

24:1 chloroform/isoamyl alcohol

5× loading dye (see recipe)

0.8% agarose gel (Voytas, 2000) prepared with 1× TAE buffer (see recipe for 50× TAE buffer)

10 mg/ml ethidium bromide stock solution: store at room temperature, protected from light

Camera suitable for gel documentation

Additional reagents and equipment for assessing DNA relaxation by eukaryotic topoisomerase I (*UNIT 3.3*), purifying and concentrating DNA (*APPENDIX 3C*), and performing agarose gel electrophoresis, ethidium bromide staining, and gel photography (Voytas, 2000)

Relax the plasmid

1. Incubate the DNA substrate with eukaryotic topoisomerase I according to the enzyme manufacturer's instructions to remove the negative supercoiling (a process termed relaxation of DNA supercoiling).

2. Assess the completeness of the relaxation reaction by agarose gel electrophoresis (see *UNIT 3.3*).

Prokaryotic topoisomerase I is less suitable for this purpose since it is inefficient at generating completely relaxed DNA. A eukaryotic type II topoisomerase may also be used.

3. Recover the relaxed plasmid from the reaction with a phenol/chloroform extraction, followed by ethanol precipitation (*APPENDIX 3C*).

CAUTION: Phenol is caustic and can cause severe burns. Solutions containing phenol should be disposed of with compatible organic waste.

Assay DNA gyrase activity

1. Set up eight reactions in 70 μ l of gyrase reaction buffer, 0.5 μ g of DNA, and 3 μ l of 28 mM ATP to achieve a titration of final NCE concentrations of 0, 10, 20, 40, 80, 160, 320, and 640 μ M. Include a separate positive control reaction using ciprofloxacin (40 to 100 μ M).

It is possible to obtain greater assay precision by testing more closely spaced NCE concentrations once an initial range of concentrations is established.

2. Add an appropriate amount of gyrase (diluted to an appropriate level of activity with gyrase dilution buffer; see annotation below) to each reaction and incubate 60 min at 25°C.

For commercial preparations, 1 unit of gyrase will catalyze the supercoiling of 0.5 μ g pBR322. Therefore, conduct initial reactions without inhibitor using 1 to 10 units of enzyme (~1 to 10 fM of purified enzyme, based on the concentration of the tetramer). Perform subsequent studies using 1 $\times$ to 2 $\times$ the concentration of enzyme needed to generate supercoiled DNA.

3. Terminate the reactions by adding 1.5 μ l of 250 mM EDTA.
4. Add 71 μ l of 24:1 chloroform/isoamyl alcohol to the reaction and centrifuge 1 min at 13,000 $\times$ g, room temperature, in a benchtop centrifuge to separate the organic and aqueous layers.
5. Add 50 μ l of the aqueous (top) phase to 10 μ l of 5 $\times$ loading dye.
6. Load samples on a 0.8% agarose gel prepared with 1 $\times$ TAE buffer. Include a control sample of gyrase reaction buffer without gyrase added to the reaction.
7. Electrophorese at 4 V/cm for 6 to 12 hr (see Voytas, 2000).
8. Stain the gel for 30 min with 1 μ g/ml ethidium bromide (diluted from 10 mg/ml stock), followed by destaining in water for 5 to 10 min.

CAUTION: Ethidium bromide is a mutagen. Wear gloves when handling, and properly dispose of all solutions.

9. Visualize the gel lanes with a transilluminator and photograph.

If no inhibition has occurred, the DNA substrate will be supercoiled and therefore run more quickly (farther) on the gel (see Fig. 3.13.4 for reactions carried out in the presence of ciprofloxacin). If inhibition has occurred, the DNA will remain relaxed and run more slowly (remaining

near the top of the gel), similar to the control DNA (reaction with no gyrase added).

Basic Protocol 3: Determination of Inhibitor Effects on DNA Cleavage by Topoisomerase II Using a Plasmid Linearization Assay

Many drugs affecting DNA topoisomerases act by stabilizing an intermediate in the enzyme reaction referred to as the cleavage or covalent complex (see Background Information). The term “covalent complex” is used to describe an interaction between topoisomerase and DNA through a phosphotyrosine linkage. The effect of topoisomerase inhibitors is to increase the amount of topoisomerase covalently bound to DNA. The procedure detailed below allows for the quantification of covalent protein-DNA complexes in cells (Liu et al., 1983). Because topoisomerases form covalent complexes with DNA, methods for measurement of protein-DNA covalent complexes correlate to studying topoisomerase poisoning (see Commentary).

The assay described in the Alternate Protocol provides a quantitative measure of drug-stabilized DNA cleavage. While it is sensitive and quantitative, it is laborious and requires the use of radioactive isotopes. The protocol detailed below uses a plasmid linearization assay and is simple and rapid. It is somewhat less sensitive than the Alternate Protocol and uses relatively large amounts of purified enzyme. The assay is derived in large part from that described by Osheroff and colleagues (Anderson et al., 1999; Burden et al., 2001). While the procedure is based on topoisomerase IV, it can be readily modified for DNA gyrase.

CAUTION: Ethidium bromide is a mutagen. Wear gloves when handling, and properly dispose of all solutions containing ethidium bromide.

Materials

- 10× topoisomerase IV reaction buffer (when using topoisomerase IV; see recipe) *or* 5× DNA gyrase reaction buffer (when using DNA gyrase; see recipe)
- Supercoiled plasmid DNA (e.g., 0.28 µg pBR322 or 0.18 µg pUC18; see Critical Parameters)
- New chemical entity (NCE) stock solution
- 1 mM positive control (e.g., ciprofloxacin from Sigma-Aldrich)
- 20 mM ATP (diluted with H₂O from purchased stock solution; store up to 6 months in small aliquots at −20°C), optional
- 4 to 20 U/µl purified topoisomerase II (DNA topoisomerase IV or DNA gyrase; TopoGen or Inspiralis; see Critical Parameters)
- 10% (w/v) SDS
- 250 mM EDTA
- 4 mg/ml stock solution of proteinase K: dilute to 0.8 mg/ml before use
- 0.8% agarose gel (Voytas, 2000) prepared with 1× TAE (see recipe for 50× TAE)
- Restriction enzyme appropriate for cutting plasmid once
- 10 mg/ml ethidium bromide stock solution: store at room temperature, protected from light
- Camera suitable for gel documentation

Additional reagents and equipment for performing agarose gel electrophoresis, ethidium bromide staining, and gel photography (Voytas, 2000)

1. Add to 1.5-ml microcentrifuge tubes (on ice) the following ingredients in the order listed:

2 μ l 10 $\times$ topoisomerase IV reaction buffer *or* 4 μ l 5 $\times$ gyrase reaction buffer

5 nM supercoiled plasmid DNA

1 to 2 μ l of NCE solution or positive control

H₂O to bring the final reaction volume to 20 μ l (including the volume of enzyme to be added in step 2).

It is most common to perform cleavage assays in the absence of ATP so that no relaxation of supercoiled plasmid will occur under these conditions. For some agents, enhanced DNA cleavage may require ATP. Therefore, for NCEs a parallel series of experiments with added ATP may be of value. In that case, use the same ATP concentrations as recommended in Basic Protocols 1 and 2.

2. Add 1 to 2 μ l of 4 to 20 U/ μ l topoisomerase II (0.1 to 1 μ g purified protein). Incubate the reaction mixture 15 to 30 min at 37°C.

The optimal incubation temperature depends on how rapidly enzyme/DNA cleavage occurs. Incubation times as short as 5 min are frequently sufficient.

3. Terminate the reaction by adding 2 μ l of 10% SDS.

It is important to add the SDS stop solution to tubes in the 37°C water bath. Do not remove the tubes to ice before adding SDS. It is especially critical to add SDS before adding the EDTA solution. Addition of EDTA prior to SDS will result in complete reversal of DNA cleavage.

4. Add 1.5 μ l of 250 mM EDTA and 2 μ l of 0.8 mg/ml proteinase K solution. Incubate 1 to 2 hr at 30°C.

The proteinase K incubation time is not critical, although sufficient time for complete digestion of the enzyme covalently bound to DNA is needed. Some prefer to incubate the proteinase K reaction at 50°C.

5. Add 2 μ l of 5 $\times$ loading dye to each sample.

6. Load the sample on a 0.8% agarose gel prepared with 1 $\times$ TAE, and electrophorese for 2 to 4 hr at 5 V/cm. Include a lane of linearized plasmid (cut with a restriction enzyme that will only cut the plasmid one time) as a control.

7. Stain the gel for 30 to 60 min in 1 μ g/ml ethidium bromide (diluted from 1 mg/ml stock), followed by destaining in water for 5 to 10 min.

CAUTION: Ethidium bromide is a mutagen. Wear gloves when handling, and properly dispose of all solutions containing ethidium bromide.

8. Visualize lanes using a UV transilluminator and photograph the gel.

Results using this assay are illustrated in Figures 3.13.5 (ciprofloxacin) and 3.13.6 (gemifloxacin and moxifloxacin).

9. Using densitometry, quantitate the concentration of linear DNA formed, typically by comparing to quantities of linear DNA concentrations formed in the presence of specific concentrations of the positive control (e.g., ciprofloxacin).

Alternate Protocol: Determination of Topoisomerase Cleavage Using the K⁺/SDS Assay

The aim of this assay is to determine the level of type II topoisomerase/DNA covalent complexes induced in vitro by an NCE by measuring the amount of radioactively labeled DNA that is covalently associated with protein. Since this occurs only when the topoisomerase is trapped as a covalent complex, this assay provides a definitive test of whether an agent is a topoisomerase II poison (causing DNA damage) as opposed to a catalytic inhibitor of enzyme activity (see Commentary for discussion). It also provides a useful and accurate measure of the potency of an agent as a poison of type II topoisomerases.

CAUTION: Radioactive materials require special handling; all supernatants must be considered radioactive waste and disposed of appropriately.

CAUTION: Ethidium bromide is a mutagen. Wear gloves when handling, and properly dispose of all solutions.

Materials

DNA substrate: plasmid DNA (e.g., pUC18 or pBR322; New England Biolabs)
*Eco*RI restriction endonuclease (New England Biolabs)
 5× loading dye (see recipe)
 0.8% agarose gel (see Voytas, 2000) prepared in 1× TAE (see recipe for 50× TAE)
 10 mg/ml ethidium bromide stock solution: store at room temperature, protected from light
 TE buffer, pH 8.0 (*APPENDIX 2A*)
 5 mM dCTP, dGTP, and dTTP
 10× Klenow buffer (provided with enzyme; New England Biolabs)
 10 mCi/ml [α -³²P] dATP (800 Ci/mmol; Amersham)
 5 U/μl Klenow fragment of DNA polymerase I (New England Biolabs)
 Sephadex G-25 spin columns (Pharmacia Biotech)
 10× topoisomerase IV reaction buffer (see recipe) *or* 5× gyrase reaction buffer (see recipe)
 New chemical entity (NCE) solutions: several concentrations diluted in H₂O
 20 to 40 U/μl purified topoisomerase IV *or* gyrase (Inspiralis; see Critical parameters)
 Topoisomerase stop solution (see recipe), 37°C (for topoisomerase) or 25°C (for gyrase)
 325 mM KCl
 Topoisomerase wash solution (see recipe)
 Scintillation fluid (Fisher Scientific)

65°C heating block

Additional reagents and equipment for performing agarose gel electrophoresis (Voytas, 2000) and purifying DNA by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (*APPENDIX 3C*)

Prepare substrate

1. Digest DNA substrate with *EcoRI* according to the restriction endonuclease manufacturer's instructions. Allot 7 µg of DNA in a volume of 20 µl for twenty reactions (see step 9).
2. Add a small portion of the restriction enzyme digestion reaction to 5× loading dye in a microcentrifuge tube.
3. Load the sample on a small 0.8% agarose gel prepared in 1× TAE. Include a lane of uncut plasmid for comparison.
4. Electrophorese 1 to 2 hr at 50 V.
5. Stain the gel 30 to 60 min in 1 µg/ml ethidium bromide (diluted from 10 mg/ml stock), followed by 5 to 10 min destaining in water.
6. Visualize the lanes with a transilluminator.

The product observed after electrophoresis will depend on the substrate. *EcoRI* digestion of pUC18 yields a single band. Since the purpose of this step is simply to verify that the restriction enzyme cut all of the DNA substrate, almost any agarose gel electrophoresis conditions are suitable. It is recommended to use a substrate that does not generate a large number of fragments following digestion with a restriction enzyme.

7. Purify the digested plasmid from the restriction digest reaction by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (see *APPENDIX 3C*).

Radioactively label substrate

1. Resuspend DNA at 0.35 µg/µl in TE buffer, pH 8.0.
2. Prepare the following 50-µl labeling mixture, adding ingredients in the indicated order:

20 µl digested DNA (7 µg)

15 µl H₂O

1 µl of 5 mM dCTP

1 µl of 5 mM dGTP

1 µl of 5 mM dTTP

5 µl of 10× Klenow buffer

5 µl of 10 mCi/ml [α -³²P] dATP 3000 Ci/mmol

2 µl of 5 U/µl Klenow fragment of DNA polymerase I.

Amounts are given for 20 reactions; the volumes may be scaled as needed.

Many kits are available for labeling with DNA polymerase I and may be used for this labeling reaction.

Incubate the reaction 30 min at 37°C.

Purify the labeled substrate from unincorporated nucleotides using a Sephadex G-25 spin column or other appropriate method.

Perform cleavage assay

1. For each NCE concentration, prepare triplicate samples. Add to 1.5-ml microcentrifuge tubes, in the order listed:
 - 2 μ l 10 $\times$ topoisomerase IV reaction buffer *or* 4 μ l of 5 $\times$ gyrase reaction buffer (depending on the enzyme used)
 - 350 ng labeled DNA substrate (from step 11)
 - 1 to 2 μ l NCE solution
 - H₂O to a final volume of 20 μ l
 - 20 U topoisomerase II (0.5 to 2 μ g purified protein).

As with Basic Protocol 3, the cleavage assays should be conducted in the absence of ATP. When cleavage assays are performed in the absence of ATP, no relaxation of supercoiled plasmid will be observed. For some agents, enhanced DNA cleavage may require ATP. Therefore, for NCEs, a parallel series of experiments with added ATP may be of value. In that case, use the same ATP concentrations as recommended in Basic Protocols 1 and 2.

Include two triplicate control reactions containing the reaction components in step 12, but omitting NCE (for one control) and omitting enzyme and NCE (for the other control).

The no-enzyme control sample is used to determine background, which is subtracted from each of the other samples. The control, including enzyme, measures drug-independent DNA cleavage.

For topoisomerase IV

- 14a. Incubate 15 min at 37°C.
- 15a. Add 1 ml of 37°C topoisomerase stop solution.

For gyrase

- 14b. Incubate 15 min at 25°C.
- 15b. Add 1 ml of 25°C topoisomerase stop solution.

It is important that the temperature of the reaction not change before the stop solution is added, as this will alter the amount of cleavage complexes. Thus, do not put the samples on ice before adding the stop solution.

Remove DNA not covalently bound to protein

1. Add 250 μ l of 325 mM KCl to each sample.
 - The addition of KCl leads precipitation of enzyme/SDS aggregates. Free DNA (i.e., DNA not covalently bound to protein) remains in solution.
2. Cool samples 10 min on ice and then microcentrifuge 10 min at 8000 $\times$ g (75% of maximum speed), 4°C.

3. Aspirate the supernatant and resuspend the pellet in 1 ml topoisomerase wash solution. Incubate the suspension 10 min at 65°C, with occasional vortexing.

CAUTION: The supernatant from this step will contain a great deal of radioactivity.

4. Repeat the washes (steps 17 and 18) twice more.
5. Microcentrifuge 10 min at $8000 \times g$ (75% of maximum speed), 4°C.

Measure radioactivity

1. Resuspend the pellet in 400 µl water at 65°C.
2. Place 100 µl of the resuspended pellet in a scintillation vial containing 4 ml scintillation cocktail and quantify the radioactivity using a scintillation counter.
3. Subtract the background (cpm from no-enzyme control) from all samples and express corrected data as cpm of sample containing NCE divided by cpm from control in the absence of NCE.

For inhibitors such as fluoroquinolones, a useful metric is the concentration of drug needed to increase the cpm precipitated by a factor of two, compared to samples without added NCE.

Basic Protocol 4: Measurement of the Minimal Inhibitory Concentration (MIC) Of Inhibitors of *E. Coli* Gyrase and Topoisomerase IV Mutants

This protocol describes a broth dilution assay for determining the minimum inhibitory concentration (MIC) of an NCE (also see UNIT 13A.3). It follows the guidelines of the National Committee for Clinical Laboratory Standards (CLSI, 2003) for determining MIC. A plating method for determining the minimum bactericidal concentration (MBC) is also provided. The assumption is that a useful property of a new topoisomerase-targeting drug may be substantial growth inhibitory activity against bacterial strains resistant to more traditional fluoroquinolones. The isogenic (genetically identical except for the mutant genes) fluoroquinolone resistant *E. coli* gyrase and topoisomerase IV mutant strains used in this protocol are those obtained from Zechiedrich (Khodursky et al., 1995) and Yoshida (Yoshida et al., 1991). These strains exhibit increased MICs for fluoroquinolone relative to the wild type strain; however, they are not defined as resistant by clinical standards. Ideally, there should be little increase in the MIC in the gyrase mutant strains as compared to the wild type strain. Similarly, the addition of a mutation in the topoisomerase IV gene to the gyrase mutant strain should not greatly increase the MIC. It may be possible that resistance to drugs that target both topoisomerase IV and gyrase would develop slowly compared to potent drugs that target only one of the two enzymes, since no single-step gyrase mutation would increase survival enough to allow selection of additional mutations.

This protocol can be readily adapted to other aerobic bacteria that grow in Mueller-Hinton media. Application to aerobic species that do not grow in Mueller-Hinton media requires the selection of appropriate growth media and may also require adjustments in incubation times and temperatures.

Materials

Mueller-Hinton broth medium (Becton Dickinson)

E. coli isogenic wild-type and mutant strains: e.g., C600 derivatives LZ23, gyrase *gyrA*^{S83L} and LZ21, *gyrA*^{S83L} *parC*^{S80L} (available from E. Lynn Zechiedrich, Baylor College of Medicine)

New chemical entity (NCE) solution to be tested

32 mg/ml ciprofloxacin (Sigma-Aldrich; reference standard antibiotic) solution

Mueller-Hinton agar plates (Becton Dickinson)

Sterile 13 × 100-mm culture tubes

250-ml Erlenmeyer flask

Grow bacterial cultures

1. Inoculate sterile 13 × 100-mm culture tubes containing 3.0 ml sterile Mueller-Hinton broth separately with each *E. coli* strain (a wild-type strain and a gyrase/topoisomerase IV mutant strain).
2. Incubate the broth cultures overnight at 37°C, without shaking.
3. Add 400 µl overnight culture to 20 ml 37°C Mueller-Hinton broth (1:50 dilution) in a 250-ml Erlenmeyer flask.
4. Incubate the cultures 30 to 90 min at 37°C, with shaking (~175 rpm), until the cells reach an OD₆₂₅ of 0.08 to 0.10, which corresponds to ~10⁸ colony forming units per ml (cfu/ml).

It is best to begin monitoring the OD at 30 min.

Prepare reference standard and NCE dilutions

1. Dispense 0.5 ml sterile Mueller-Hinton broth into a sufficient number of sterile 13 × 100-mm culture tubes for each strain and dilution to be tested.

Fifteen test tubes will be needed for each bacterial strain tested.

2. Dispense an additional 0.5 ml sterile Mueller-Hinton broth to one of the test tubes for each bacterial strain to be tested (1 ml medium, total).

This tube will be a positive bacterial growth control, with no antibiotic solution added.

3. Dispense an additional 1.5 ml sterile Mueller-Hinton broth to one of the test tubes for each bacterial strain to be tested (2 ml medium, total).

This tube will serve as a negative control, with no bacteria or antibiotic added.

4. For the first ciprofloxacin or NCE dilution test tube (0.5 ml medium), add 48 µg of antibiotic or NCE from the stock solutions, and then add additional sterile Mueller-Hinton broth to bring the final volume to 1.5 ml.

This test tube should now be at a final concentration of 32 µg/ml for the test agent.

5. Make serial 2-fold dilutions of the original ciprofloxacin or NCE dilution tube: Pipet 0.5 ml of the 32 µg/ml antibiotic dilution to the next test tube. Continue pipetting 0.5 ml successively for the remaining test tubes, ending with 0.001 µg/ml of the ciprofloxacin or NCE in the final tube. Discard 0.5 ml from the final tube.

The series will contain ciprofloxacin or NCE at final reaction concentrations of 16, 8, 4, 2, and 1 µg/ml, followed by 500, 250, 125, 62.5, 31.25, 15.6, 7.8, and 3.9 ng/ml after adding the bacteria.

Determine MIC

1. Dilute the 10^8 cfu/ml culture 1:100 in sterile Mueller-Hinton broth and add 1.0 ml of this dilution to each set of NCE dilution tubes and the positive control tube containing no antibiotic or NCE.

The final bacterial concentration in each tube will be 5×10^5 cfu/ml.

2. Incubate the tubes 16 to 20 hr at 37°C, without shaking.
3. Visually inspect the tubes for bacterial growth.

The MIC is the lowest concentration of agent that completely inhibits the growth of the bacteria. For the test to be valid, the negative control tube must not show any visible growth, and the positive control must have visible growth.

Determine MBC

1. Shake the tubes from step 12 for 1 hr at room temperature to ensure that viable cells do not adhere to the sides of the tubes.
2. Serially dilute the growth control tubes: Add 10 µl of the culture to 990 µl Mueller-Hinton broth (10^{-2} dilution). Add 10 µl of the 10^{-2} dilution to 90 µl Mueller-Hinton broth (10^{-3} dilution). Repeat once more to prepare a 10^{-4} dilution.
3. Transfer 100 µl of the diluted cultures from step 14 to a sterile Mueller-Hinton agar plate and spread using a sterile glass spreader (see UNIT 13A.3 for spreading technique).
4. Transfer 100 µl (no dilution) of each broth cultures from the test tubes that showed no growth onto sterile Mueller-Hinton agar plates and spread these cultures using sterile glass spreaders.
5. Incubate the plates overnight at 37°C. Count the colonies on all of the plates.

The minimal bactericidal concentration (MBC) is the concentration of NCE at which 99.9% of bacteria are killed. This is equal to a ≥ 3 log₁₀ reduction in cfu.

Basic Protocol 5: Measurement of the Mutant Prevention Concentration (MPC)

The mutant prevention concentration (MPC) is defined as the concentration of NCE required to prevent the growth of spontaneous mutants. While this concentration is often higher than the MIC, the difference between the MIC and MPC varies depending on the NCE and the bacterial strain used for testing (Linde and Lehn, 2004; Marcusson et al., 2005). An NCE that has an MPC close to the MIC may be less likely to result in development of resistant isolates because it will not allow the growth of single-step mutants (see Olofsson et al., 2006).

Materials

Mueller-Hinton broth (Becton Dickinson): prepare according to the manufacturer's directions

Mueller-Hinton powdered medium (Becton Dickinson)

New chemical entity (NCE) stock solution

E. coli K12 wild-type strain: e.g., C600; American Type Culture Collection (ATCC) #23724

E. coli National Committee for Clinical Laboratory Standards (CLSI) quality control strain: ATCC #25922

Sterile 13 × 100-mm culture tubes

250-ml Erlenmeyer flasks

1. Inoculate sterile 13 × 100-mm culture tubes containing 3 ml sterile Mueller-Hinton broth separately with the wild type and quality control *E. coli* strains.
2. Allow the cultures to incubate overnight at 37°C, without shaking.
3. Meanwhile, prepare Mueller-Hinton agar plates from powdered medium according to the manufacturer's instruction and add NCE stock solution after the medium has cooled to 55°C to obtain a range of NCE concentrations (e.g., 0.1 to 100 µM).
4. Add 100 µl of the overnight culture to a 250-ml flask containing 100 ml sterile 37°C Mueller-Hinton broth.

One 100-ml culture will be used to inoculate ten plates.

5. Incubate this culture at 37°C, with shaking at 175 rpm, until reaching a concentration of 10⁹ cells/ml (OD₆₀₀ = 1.0).
6. Centrifuge 10-ml aliquots 10 min at 3000 × g, room temperature.
7. Discard the supernatant and resuspend the cells in 50 µl Mueller-Hinton broth by gentle pipetting.
8. Plate the entire 50 µl of resuspended cells on one Mueller-Hinton agar plate with a defined concentration of NCE.
9. Incubate the agar plates 48 hr at 37°C, checking the plates for colonies at 24 and 48 hr.

The MPC is the lowest antibiotic concentration at which no colonies grow on the agar.

Basic Protocol 6: In Vivo Inhibition of Supercoiling Activity of Gyrase

A unique property of gyrase is its ability to induce negative supercoiling in DNA (see Wang 1985, 1996 for an introduction to topics related to supercoiling). Measuring inhibition of gyrase supercoiling can aid in determining the potency of new gyrase-targeting agents. With the assay described below it is possible to determine the in vivo inhibition of gyrase supercoiling activity by topoisomerase-inhibiting compounds. In the absence of an inhibitor, gyrase adds negative supercoiling in plasmid DNA. However, upon addition of a gyrase-inhibiting NCE (at an effective concentration) the enzyme no longer functions, and the extracted plasmids will be in a relaxed state. The suggested NCE test concentrations are based upon fluoroquinolone MICs for the bacteria used in the assay. A more precise measurement may be made by performing additional dilutions once the general range of inhibition is established. The conditions for this assay are based on those established for *E. coli*. Relevant conditions for other organisms may differ substantially, and must be determined empirically.

Materials

Bacterial strain encoding resistant topoisomerase IV (e.g., *parC*^{L80}; available from E. Lynn Zechiedrich, Baylor College of Medicine)

Plasmid DNA (e.g., pUC18, pBR322, or any plasmid that carries a selectable marker such as ampicillin resistance)

Mueller-Hinton broth media (Becton Dickinson) with and without antibiotic (appropriate for selection of the plasmid used)

New chemical entity (NCE)

5× loading dye (see recipe)

0.8% agarose gel (Voytas, 2000) prepared with 1× TAE buffer (see recipe for 50× TAE)

10 mg/ml ethidium bromide stock solution: store at room temperature, protected from light

Sterile 26 × 250-mm test tubes

Camera for gel documentation

Additional reagents and equipment for transforming bacteria using CaCl₂ or electroporation (Seidman et al., 1997), purifying DNA by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (APPENDIX 3C), and performing agarose gel electrophoresis, ethidium bromide staining, and gel photography (Voytas, 2000)

1. Transform the bacterial strain with the plasmid DNA by either CaCl₂ chemical transformation or electroporation.

The transformed bacterial strain can be stored at −80°C as a 15% glycerol stock.

2. Inoculate 3 ml of sterile Mueller-Hinton broth containing appropriate selection antibiotic with the transformed strain. Incubate overnight at 37°C, without shaking.
3. Dilute NCE in sterile Mueller-Hinton broth to final concentrations of 0.0, 0.005, 0.01, 0.02, 0.04, 0.08, and 0.16 µg/ml.
4. Add 10 ml of each dilution to individual sterile 26 × 250-mm test tubes and inoculate each tube with 200 µl of the overnight bacterial culture.
5. Incubate at 37°C, with shaking at 175 rpm, until the culture reaches an OD₆₀₀ of 0.4.
6. Isolate the plasmid DNA from each culture (APPENDIX 3C).
7. Add 25 µl (typically, 50 to 100 ng) isolated plasmid DNA to 6 µl 5× loading dye.

DNA yields may vary considerably. One may wish to quantify the miniprep DNA.

8. Load the samples on a 0.8% agarose gel prepared with 1× TAE buffer and electrophorese for 16 hr at 4 V/cm (see Voytas, 2000).
9. Stain the gel for 30 min with 1 µg/ml ethidium bromide, followed by destaining in water for 5 to 10 min. View the lanes with a transilluminator and photograph the gel (see Voytas, 2000).

If no inhibition has occurred, the plasmid will be supercoiled and will run farther on the gel. If inhibition has occurred, the plasmid will be more relaxed and will run slower on the gel. The DNA should run similar to the no-drug control if no inhibition has occurred.

Reagents and Solutions

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Gyrase dilution buffer

0.2 M potassium phosphate buffer, pH 6.8 (see APPENDIX 2A)

1 mM Na₃ EDTA (see APPENDIX 2A)

1 mM dithiothreitol

10% (w/v) glycerol

3.6 mg/ml bovine serum albumin

Divide into small aliquots and store up to 6 months at −20°C

This buffer is used when the gyrase preparation is more concentrated than convenient for use. This buffer is also appropriate for diluting topoisomerase TV.

Gyrase reaction buffer, 5×

175 mM Tris-HCl, pH 7.5 (see APPENDIX 2A)

30 mM MgCl₂

9 mM spermidine-HCl

120 mM KCl

45 µg *E. coli* tRNA per ml

1.8 mg/ml bovine serum albumin

Divide into small aliquots and store up to 6 months at −20°C

Loading dye, 5×

30% (v/v) glycerol

0.25 mg/ml bromphenol blue

Store up to 1 year at 4°C

As an alternative, almost any commonly used loading dye can be used for these assays. Loading dyes are also readily available commercially.

TAE, 50×

For 1 liter:

242 g Tris base

100 ml of 0.5 M EDTA, pH 8.0 (see APPENDIX 2A)

57.1 ml glacial acetic acid

Add enough H₂O to dissolve solids, adjust pH with HCl to 7.6–7.8, and then adjust the final volume to 1000 ml.

Store indefinitely at room temperature.

For a 1× solution, add 20 ml 50× TAE to 980 ml water and mix well.

TAE is stable indefinitely either as a 1× or 50× solution.

Topoisomerase TV reaction buffer, 10×

350 mM Tris-Cl, pH 7.9 (see APPENDIX 2A)

100 mM MgCl₂
 50 mM DTT
 500 µg/ml bovine serum albumin
 Divide into small aliquots and store up to 6 months at –20°

Topoisomerase stop solution

1.25% (w/v) SDS
 5 mM EDTA, pH 8.0 (see APPENDIX 2A)
 0.4 mg/ml salmon sperm DNA
 Store up to 6 months at room temperature

Topoisomerase wash solution

10 mM Tris-Cl, pH 8.0 (see APPENDIX 2A)
 100 mM KCl
 1 mM EDTA (see APPENDIX 2A)
 1 mg/ml salmon sperm DNA
 Store up to 6 months at 4°C

Commentary

Background Information

Topoisomerase inhibitors active against prokaryotic enzymes are important antibacterial agents, and agents active against eukaryotic enzymes are potential anti-cancer agents. While earlier literature distinguishes between bacterial and eukaryotic enzymes, it is now clear there are many similarities, as well as important differences, between enzymes from these two kingdoms. For example, the type IA enzymes were originally described as bacterial type I topoisomerases. Subsequently it was shown that virtually all organisms, prokaryotic and eukaryotic, have one or more type IA topoisomerase. Similarly, the first eukaryotic type I topoisomerases were found to be mechanistically distinct from type IA topoisomerases, and were therefore termed type IB. Although absent from some of the more common organisms, including *E. coli*, *S. typhimurium*, or *B. subtilis*, type IB topoisomerases have been recently identified in other bacteria (Krogh and Shuman, 2002).

Mechanism of action of topoisomerase poisons—Therapeutically relevant agents targeting topoisomerases have a unique mechanism of action. In the normal topoisomerase reaction cycle, type II enzymes bind to and cleave DNA by forming a covalent complex with it. Key tyrosine residues attack the phosphodiester backbone of DNA, forming a phosphotyrosine bond. The enzyme then passes DNA strands through the break, resulting in relaxation of DNA supercoiling, introduction of supercoils (by DNA gyrase, see Fig. 3.13.1), or decatenation of catenated DNA. After strand passage, the energy of the phosphotyrosine bond is used to restore the phosphodiester backbone of DNA. Prokaryotic type II topoisomerases are A₂B₂ heterotetramers. For DNA gyrase, the gyrA subunit contains the active site tyrosine that cleaves DNA, and for topoisomerase IV, the parC subunit contains the active site tyrosine.

Topoisomerase poisons—agents that cause direct cytotoxic damage (more important for drugs targeting the eukaryotic enzyme) rather than simply inhibiting essential enzyme

activity—trap the covalent enzyme-DNA complex, resulting in DNA damage that includes DNA double-strand breaks and protein covalently attached to DNA. Covalent complexes are quantified in two ways: by measuring the levels of protein covalently bound to DNA or by directly assaying for DNA strand breaks in the presence of topoisomerase and drug. Basic Protocol 3 allows detection of double-strand breaks on a circular DNA substrate. The Alternate Protocol directly measures protein covalently bound to DNA by selective isolation of DNA covalently attached to protein. Since formation of a covalent complex is a normal step in the topoisomerase reaction, double-strand breaks are observed even in the absence of a topoisomerase poison. This is particularly true for *E. coli* topoisomerase IV (Anderson et al., 1998). However, addition of a topoisomerase poison leads to greatly increased levels of covalent complexes.

Inhibition of enzyme activity can be readily observed with potent bacterial topoisomerase II poisons, such as fluoroquinolones. This differs from poisons that act against eukaryotic topoisomerases (e.g., the anti-cancer drug etoposide), where inhibition of relaxation is less pronounced. Therefore, inhibition of enzyme activity, as described in Basic Protocols 1 and 2, is a commonly used assay for studying fluoroquinolones, but is less commonly used as an assay for drugs targeting eukaryotic topoisomerase II. Even though inhibition of enzyme activity is a useful assay for the prokaryotic enzymes, it is clear that the most important determinant of inhibitor action is the ability to generate enzyme-mediated DNA damage (Hooper, 2000; Anderson and Osheroff, 2001; Drlica and Malik, 2003). For example, in cells expressing both a drug-sensitive and a drug-resistant enzyme, sensitivity is dominant over resistance. This occurs because the enzyme-mediated DNA damage is responsible for lethality, with the DNA damage occurring even when a drug-resistant enzyme is present (provided that a drug-sensitive enzyme is also present). This effect may also be due in part to the generation of heterodimers between drug-sensitive and drug-resistant enzymes, so a contribution of inhibition of enzyme activity cannot be excluded. Several papers have carefully discussed this critical point (Kreuzer and Cozzarelli, 1979; Froelich-Ammon and Osheroff, 1995).

Resistance to fluoroquinolones—Resistance to the fluoroquinolones occurs by several different mechanisms. Mutations in the genes that encode the target topoisomerases, especially *gyrA*, encoding the GyrA subunit of DNA gyrase, and *parC*, encoding the ParC subunit of topoisomerase IV, increase the minimum inhibitory concentration (MIC) of fluoroquinolones by reducing the susceptibility of the enzyme to the inhibitor (Takahata and Nishino, 1988; Khodursky et al., 1995; Hooper, 2001; Drlica and Malik, 2003). These mutations occur in a region near the active site tyrosine termed the quinolone resistance determining region. However, mutations in *gyrB* (or *parE*) can also lead to quinolone resistance, although usually to lower levels than for *gyrA* or *parC* mutations. At present, there are no reported or solved three dimensional structures of a type II topoisomerase bound to an inhibitor, so the molecular details of fluoroquinolone action remain unknown.

A second mechanism, increased levels of efflux pumps capable of transporting a variety of small molecules, also increases the MIC of certain fluoroquinolones, presumably by altering intracellular concentrations of the drug, but additional physiological roles for the pumps may also contribute to drug resistance (Drlica and Malik, 2003; Payot et al., 2004; Yang et al., 2006).

A third resistance mechanism may be particularly relevant clinically since it is a plasmid-borne resistance mechanism. These plasmid-mediated resistance mechanisms lead to chemical modification of some fluoroquinolones, e.g., acetylation of the piperazinyl amine on ciprofloxacin and norfloxacin (but not enrofloxacin, gemifloxacin, levofloxacin, or pefloxacin (Robicsek et al., 2006)

Since both topoisomerase IV and DNA gyrase are fluoroquinolone targets, an important question is which enzyme is the most important cytotoxic target. The answer appears to depend on the bacteria under examination. In Gram-negative bacteria such as *E. coli*, fluoroquinolones preferentially kill cells through their action on DNA gyrase, with topoisomerase IV as a secondary target. In contrast, for most Gram-positive bacteria, topoisomerase IV is the primary cytotoxic target, while gyrase is a secondary target (Hooper, 2000). One exception is *Streptococcus pneumoniae*, which appears to have different primary targets, depending upon the particular drug.

One hypothesis for the differences in targets between Gram-negative and Gram-positive bacteria is based on the higher in vitro sensitivity of DNA gyrase in Gram-negative bacteria to fluoroquinolones (Hooper, 2000, 2001; Anderson and Osheroff, 2001). The prediction is that the enzyme that is most sensitive to a specific fluoroquinolone (in this case gyrase) is the primary drug target, and the effects on the secondary target (in this case topoisomerase IV) would not be observed unless the primary target were less susceptible than the secondary target (e.g., because of mutations that reduce susceptibility to the drug; Hooper, 2000, 2001). However, for Gram-negative bacteria, the difference in the in vitro susceptibility to many fluoroquinolones between gyrase and topoisomerase IV is less than two-fold, which may be insufficient to explain differences in the cytotoxic effects between the targets (Anderson and Osheroff, 2001).

An alternative hypothesis is that the differences in the ability of different topoisomerases to act as cytotoxic targets may stem from different biological functions. For example, if a topoisomerase acts ahead of a replication fork, the potential cytotoxic consequences will be more extreme than if the enzyme acts behind the replication fork (Nitiss, 1994). Interestingly, in *Streptococcus pneumoniae*, the primary target depends upon the particular fluoroquinolone being tested. Fisher (see Pan et al., 2001) has examined the difference between ciprofloxacin and Sparfloxacin targeting in *S. pneumoniae*. While genetic studies revealed that topoisomerase IV was the primary target for ciprofloxacin, and gyrase was the primary target for Sparfloxacin, in vitro inhibitory activity was similar for the two drugs for both enzymes. Fisher hypothesizes that additional proteins, such as helicases or polymerases, may block the quinolone binding sites on the target enzymes, or that the differences may be due to drug-specific differences in the ternary complex conversion to lethal DNA adducts (Pan et al., 2001).

Mechanistic considerations of how fluoroquinolones target type II topoisomerases and details of cell killing mechanisms are important questions, the answers to which will help guide the development of new generations of agents targeting gyrase and/or topoisomerase IV. The continuing evolution of drug-resistant bacteria requires the continuous development of new drugs. Fluoroquinolones have been successful because of the ability to synthesize new, more potent variations. Although a diverse group of fluoroquinolones has been used clinically (see Fig. 3.13.2 for examples), it is clear that other approaches will be required if topoisomerases are to remain a useful antibacterial target. For example, it would be of great value to find new type II topoisomerase poisons, particularly new chemotypes that are active against fluoroquinolone-resistant bacteria. Such agents must be highly potent, selective (i.e., inactive against eukaryotic topoisomerase II, which is biochemically closely related to the prokaryotic enzymes; Strumberg et al., 2002), and have useful pharmacokinetic properties.

Another potential approach is the identification of agents that target topoisomerase II in different ways. For example, it may be possible to identify active catalytic inhibitors of topoisomerase II that do not trap covalent complexes (Flatman et al., 2005, 2006). Since type II topoisomerases are essential for viability in all bacteria, the development of catalytic inhibitors may result in an activity profile distinct from the fluoroquinolones. The assays

described in this unit should be useful in helping to determine whether these conceptual approaches can lead to new antibiotics.

Critical Parameters and Troubleshooting

Table 3.13.3 describes some problems commonly encountered using the assays described in this unit, along with possible reasons for the problems and suggestions for overcoming or avoiding them.

Sources of purified enzymes for Basic Protocols 1, 2, and 3 and the Alternate Protocol—Basic Protocols 1, 2, and 3 require purified topoisomerases, either DNA gyrase or DNA topoisomerase IV. The success of the experiments depends on reliable enzyme preparations. Two commercial sources of purified prokaryotic type II topoisomerases (both DNA gyrase and topoisomerase IV) are available, TopoGen (see *SUPPLIERS APPENDIX*) and Inspiralis (<http://www.inspiralis.com>; contact Mrs. Alison Howells). It should be noted that the assay buffers recommended by the manufacturer may differ from those described in this unit; in general, use the manufacturer's assay buffers with enzymes provided by them.

Many purification schemes for DNA gyrase (Mizuuchi et al., 1978; Hallett et al., 1990; Maxwell and Howells, 1999) and topoisomerase IV (Kato et al., 1992; Anderson et al., 1999; Peng and Mariani, 1999) have been described, especially for enzymes from *E. coli*, *Bacillus*, and other common laboratory genera. In some cases, it may be desirable or necessary to purify bacterial type II topoisomerases from other bacteria, or to characterize mutant enzymes. The details of such experiments depend heavily on the bacterial protein under examination. In general, the simplest strategy is to isolate the genes encoding the relevant topoisomerase by PCR (Huang, 2001), followed by overexpression and purification in *E. coli*.

Regardless of the source of the enzymes, they must be handled appropriately for the assay to work properly. In general, purified enzymes are stored in small portions at -80°C , and multiple freeze-thaw cycles should be avoided. Enzymes maintain activity best when stored at relatively high concentrations (typically at the concentration provided by the manufacturer). Enzyme dilution buffers are usually provided by the supplier. For the sake of consistency, it is important to be wary of results obtained with preparations that have markedly different enzyme activities.

The availability of a large number of active fluoroquinolones provides a ready source for positive controls. Ciprofloxacin is commonly used as a positive control and is recommended for this purpose. Assays for drug sensitivity in vivo and in vitro should be positive with this agent. For example, a failure to observe inhibition of decatenation in the presence of ciprofloxacin in the test strains, which do exhibit a degree of sensitivity to ciprofloxacin, suggests a problem with the assay rather than inherent resistance.

Supercoiled plasmid DNA for Basic Protocols 1, 2 and 3 and the Alternate Protocol—Basic Protocols 1, 2, and 3 and the Alternate Protocol require purified supercoiled plasmid DNA. The plasmid can be chosen for the convenience of the investigator. High-copy plasmids such as pUC18 are typically a good choice. Standard plasmid preparation assays or kits that provide good quality DNA suitable for subcloning are also appropriate for the assays described here.

Anticipated Results

Interpretation of in vitro enzyme assays in the presence of inhibitors—The assays described in Basic Protocols 1 and 2 measure the ability of agents to interfere with

topoisomerase II reactions. As noted in those protocols, the decatenation assay is most appropriate for determining the activity of an agent against topoisomerase IV, while inhibition of supercoiling is the most relevant assay for DNA gyrase. The assays provide information about the susceptibility of the *E. coli* enzymes. It may also be desirable to examine topoisomerases and gyrases from other bacteria, which will require obtaining the enzymes from noncommercial sources. A limitation of Basic Protocols 1 and 2 is that they do not reveal whether an inhibitor is a Top2 poison. For this question, Basic Protocol 3 or the Alternate Protocol is required.

Basic Protocols 1 and 2 describe enzyme inhibition assays, and the data obtained are the gel electrophoresis photographs of enzyme activity. For example, in Figure 3.13.3, partial inhibition of decatenation (see Basic Protocol 1) is seen at 10 μ M ciprofloxacin, with complete inhibition at 40 μ M ciprofloxacin. Similarly, in Figure 3.13.4, partial inhibition of supercoiling is seen at 8 μ M ciprofloxacin, and complete inhibition is seen at 40 μ M ciprofloxacin (see Basic Protocol 2). In Figure 3.13.5, linear (form III) DNA is seen at ciprofloxacin concentrations in the range of 0.1 to 0.25 μ M (see Basic Protocol 3). Note that the ciprofloxacin concentrations required for inhibition of enzyme activity differ from the concentrations needed to generate double strand breaks in DNA. The relationship of drug concentrations to in vitro enzyme inhibition and in vivo cytotoxicity represents an important experimental question for developing new drugs targeting bacterial topoisomerases.

Interpretation of in vivo assays—Basic Protocol 4 describes a reliable method for determining the MIC of an NCE. The technique provides accuracy within a factor of two because two-fold differences in drug concentration are made. If a more precise measurement is required, additional drug concentrations may be tested in a subsequent assay. By comparing results obtained using an NCE to those obtained using ciprofloxacin or another reference compound, the relative efficacy of the NCE can be inferred. Importantly, the assay can be readily applied to bacterial strains that are resistant to fluoroquinolones, allowing identification of agents that can overcome mutations in gyrase or topoisomerase IV that confer drug resistance.

A measure of the ability of bacteria to become drug-resistant is obtained using Basic Protocol 5. Often, a plateau is observed in the mutant selection window, with colonies appearing on several drug concentrations higher than the MIC and then decreasing again. This may be due to the MICs of the first step mutants. At concentrations higher than the MICs of the first step mutants, colonies should not be readily observed because resistance would require two simultaneous beneficial mutations (Drlica and Zhao, 2007). A significant limitation of this assay is that it is unclear whether measuring the MPC in vitro correlates with the development of resistance in vivo. In vitro, the drug concentration on the plate will remain constant for the entire duration of the experiment. However, during an actual treatment the drug concentration in the body is high initially and then drops (Drlica and Zhao, 2007). Thus, mutant selection may occur differently in vivo.

Using the methods described in Basic Protocol 6 provides data relating to the in vivo efficacy of a drug as a DNA gyrase inhibitor. The assay has been applied mainly to *E. coli*, and directly demonstrates drug effects in vivo. A limitation of this assay may be that the information does not translate to other bacterial species. This assay can be adapted to other bacteria that have autonomously replicating plasmids.

Time Considerations

Basic Protocols 1, 2, and 3 can each be completed in 1 day, depending on the time allotted for electrophoresis. Note that electrophoresis can be performed at higher voltage, although with some loss of resolution. This allows a quick check of enzyme activity (important to

verify that a commercial enzyme preparation is active prior to starting a large experiment), and completion of the biochemical assays (typically ~2 hr) in 1 day. If a large series of assays is being conducted over several days, it is not necessary to check enzyme activity every day.

The Alternate Protocol requires 1 to 2 days with substantial hands-on time. In particular, the washing procedure typically takes ~2 to 3 hr.

Basic Protocol 4 requires 3 days to determine the MIC, with an additional day if the MBC is quantified. The first day will only require ~5 min preparation time. The second day will be the most labor intensive. Depending upon how many different drugs and concentrations are tested, 1 to 2 hr may be needed to prepare the dilutions. The third day will be the least time-intensive because the test tubes are simply analyzed for the presence or absence of turbidity.

Basic Protocol 5 requires 4 days, with only day 2 requiring significant hands-on time. Several hours may be needed to process samples, depending on the number being tested.

Basic Protocol 6 can be completed in two days once the appropriate bacterial strain is made. Note that the bacterial strain can be stored at -80°C as a 15% glycerol stock, and need not be freshly transformed each time.

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Key References

- Anderson et al., 1998. See above. A fine detailed description concerning mechanistic questions of inhibiting bacterial topoisomerases.
- Hooper, 2001. See above. The importance of fluoroquinolones has led to a large literature on these agents. These two references are a fine starting point.
- Drlica and Malik, 2003. See above. This review covers basic biochemical and in vivo mechanisms concerning how fluoroquinolones target bacteria.

Figure 3.13.1.

Key reactions of bacterial type II topoisomerases. Type II topoisomerases (DNA gyrase and topoisomerase IV) carry out their reactions by introducing transient DNA double-strand breaks. **(A)** During DNA replication, DNA gyrase converts relaxed covalently closed (circular) DNA to DNA that is negatively supercoiled. The solid line represents a DNA double helix; DNA gyrase reduces the winding of the DNA double helix (formally the linking number). In order to maintain Watson-Crick base pairing, the DNA double helix wraps around itself, forming a more compact structure. **(B)** At the completion of DNA replication, the two replicated molecules are partly wrapped around each other. For circular molecules, the products of replication are called catenanes. Type II topoisomerases are required to separate the catenated (linked) molecules. While both DNA gyrase and other type II topoisomerases can cause decatenation (separation of the DNA circles) in vitro, topoisomerase IV in *E. coli*, (a type II topoisomerase that cannot introduce negative supercoils) is thought to be the principal decatenase.

Figure 3.13.2.

Structures of fluoroquinolones. The basic quinolone structure, without modifications, is shown with the ring atoms numbered (top center). The structures of several fluoroquinolones (norfloxacin, ciprofloxacin, moxifloxacin, and levofloxacin) are shown as examples of side group substitutions.

Figure 3.13.3.

Inhibition of topoisomerase IV-catalyzed decatenation of kinetoplast DNA by ciprofloxacin. The standard decatenation reaction mixtures containing 0.3 μg of kinetoplast DNA (kDNA;TopoGen), 50 fmol (as tetramer) of *E. coli* topoisomerase IV, and indicated concentrations of ciprofloxacin (cipro) were incubated, processed, and analyzed as described in Basic Protocol 1. Partial inhibition of decatenation is seen at 10 μM ciprofloxacin, and complete inhibition is seen at 40 μM ciprofloxacin. DNA is quantified in the decatenated band. Quantitation of DNA left in the upper bands near the wells is unreliable because catenated DNA does not effectively enter the gel.

Figure 3.13.4.

Inhibition of DNA gyrase-catalyzed supercoiling by ciprofloxacin. The supercoiling reaction mixtures containing 0.3 μg of pBR322 relaxed DNA and 10 fmol (as tetramer) of *E. coli* DNA gyrase were incubated with the indicated concentrations of ciprofloxacin (cipro), and the DNA products were analyzed by agarose gel electrophoresis (see Basic Protocol 2 for details). Partial inhibition of supercoiling is seen at 8 μM ciprofloxacin, and complete inhibition is seen at 40 μM ciprofloxacin.

Figure 3.13.5.

DNA cleavage by *S. aureus* topoisomerase IV in the presence of ciprofloxacin. Double-stranded DNA cleavage by topoisomerase IV converts covalently closed plasmid form I DNA (FI) to linear form III molecules (FIII). The cleaved plasmid DNA also has covalently bound protein, which is digested with proteinase K prior to electrophoresis. The position of nicked circular form II DNA (FII) is also shown. Note that a substantial amount of linear DNA is seen at ciprofloxacin concentrations well below those needed to completely inhibit decatenation. Figure is reproduced with permission from Anderson et al. (2000).

Figure 3.13.6.

DNA cleavage by *S. pneumoniae* DNA gyrase in the presence of gemifloxacin and moxifloxacin. As in Figure 3.13.5, this gel shows double-stranded DNA cleavage by a type II topoisomerase (*S. pneumoniae* DNA gyrase), converting covalently closed plasmid form I DNA (FI) to linear form III molecules (FIII). The cleaved plasmid DNA (pBR322) also has protein covalently bound, which is digested with proteinase K prior to electrophoresis. Lane a shows the substrate DNA, a mixture of supercoiled (FI) and nicked, circular DNA (FII). Lane b shows the migration of DNA linearized with a restriction enzyme (FIII). The concentrations of gemifloxacin and moxifloxacin are indicated. This figure is reproduced with permission from Yague et al. (2002).

The Topoisomerases of *E. coli*^a

Table 3.13.1

Enzyme	Genes	Type	Relaxes negatively supercoiled DNA?	Relaxes positively supercoiled DNA?	Introduces supercoils into DNA?
Topoisomerase I	<i>topA</i>	IA	Yes	No	No
DNA gyrase	<i>gyrA, gyrB</i>	IIA	Yes ^b	Yes	Yes
Topoisomerase III	<i>topB</i>	IA	Yes	No	No
Topoisomerase IV	<i>parC, parE</i>	IIA	Yes	Yes	No

^a*E. coli* lacks a type IB topoisomerase; however, some bacterial species have type IB topoisomerases.

^bRelaxation of negatively supercoiled DNA requires special conditions.

Table 3.13.2
The Known Topoisomerases in Mammalian Cells

Enzyme	Yeast homolog	Type	Relaxes negatively supercoiled DNA?	Relaxes positively supercoiled DNA?	Introduces supercoils into DNA?
Top1	yTOP1	IB	Yes	Yes	No
Top1 Mt	None	IB	Yes	Yes	No
Top2 α	yTOP2	II	Yes	Yes	No
Top2 β	None	II	Yes	Yes	No
Top3 α	yTOP3	IA	Yes	No	No
Top3 β	None	IA	Yes	No	No
hSpo11 ^a	Spo11	IIIB	No	No	No

^aSpo11 is required for meiotic development and is homologous to type IIB topoisomerases, but has not been shown to have topoisomerase activity (type IIB topoisomerases have been found in Archaeobacteria and in plants).

Table 3.13.3
Troubleshooting for Bacterial Topoisomerase Inhibition Assays

Problem	Possible cause	Solution
<i>Basic Protocol 1</i>		
No detected relaxation of supercoiled DNA in samples lacking drug	Loss of enzyme activity	Use a fresh aliquot of enzyme
No detected relaxation of supercoiled DNA in samples lacking drug	Degradation of ATP (a common issue with these assays)	Use freshly prepared ATP
<i>Basic Protocol 2</i>		
No detected decatenation in samples lacking drug	Loss of enzyme activity	Use a fresh aliquot of enzyme
No detected decatenation in samples lacking drug	Degradation of ATP (a common issue with these assays)	Use freshly prepared ATP
<i>Basic Protocol 3</i>		
No detected DNA cleavage in samples containing positive control	Loss of enzyme activity	Use fresh aliquot of enzyme and verify (e.g., using Basic Protocols 1 or 2) the quantitative level of enzyme activity matches manufacturer's specification
<i>Alternate Protocol</i>		
High levels of counts in no-DNA samples	Incomplete washing	Add a wash step; take care to remove all supernatant
No dose-dependent increase in DNA cleavage	Agent binds to DNA	Try lower NCE concentrations (result not necessarily indicative of problems with experimental procedures)
No dose-dependent increase in DNA cleavage, with large sample-to-sample variability	Incomplete washing	Add a wash step; take care to remove all supernatant
<i>Basic Protocol 4</i>		
No growth in drug culture test tubes when expected (based upon the control)	NCE stock concentration too high	Recalculate amount of NCE and solute required; carefully measure NCE powder and solute
	Bacteria not added/insufficient bacteria added	Repeat experiment, carefully adding bacteria; ensure appropriate bacterial concentration used
Growth observed in NCE tube when not expected (based upon the control)	NCE stock concentration too low	Recalculate amount of NCE and solute required; carefully measure NCE powder and solute
	Too many bacteria added	Ensure appropriate bacterial concentration used