A T5 Exonuclease-Based Assay for DNA Topoisomerases and DNA Intercalators

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ABSTRACT: DNA topoisomerases, essential enzymes to all living organisms, are important targets of certain antibiotics and anticancer drugs. Although efforts have been taken to identify new inhibitors targeting DNA topoisomerases, limited high throughput screening (HTS) studies have been conducted since a widely accessible HTS assay is not available. We report here the establishment of a fluorescence-based, low-cost HTS assay to identify topoisomerase inhibitors. This HTS assay is based on a unique property of T5 exonuclease that can completely digest supercoiled plasmid pAB1 containing an “AT” hairpin structure and spare relaxed pAB1 and has been validated by screening a small library that contains 50 compounds for various topoisomerases. This T5 exonuclease-based HTS assay can also be used to identify DNA intercalators, the major false positives for identifying topoisomerase inhibitors using this HTS assay. Additionally, we found a new compound that potently inhibits human and bacterial DNA topoisomerase I.

INTRODUCTION

DNA supercoiling or topology is a property deeply rooted in the DNA double helix.1 Typically, naturally occurring DNA is (−) supercoiled (sc).1−3 Free energy constrained in the (−) supercoiling of DNA molecules greatly promotes a number of essential DNA processes, such as DNA replication, DNA recombination, and transcription.1,3 For instance, all stages of DNA replication have topological issues.1,2,3,4 Usually, (−) supercoiling favors the formation of functional initiation complex.5−10 In some cases, DNA replication initiation depends on (−) supercoiling.11 Without removing the (+) supercoils by a DNA topoisomerase, DNA replication forks would be arrested.12 For DNA replication termination, the newly replicated daughter DNA molecules would form catenated DNA and not separate into individual daughter DNA molecules without the assistance of a DNA topoisomerase.12−15 These examples demonstrate the essentiality of DNA topology and topoisomerases. Because of their importance to living organisms, DNA topoisomerases are the targets of certain clinically important antibiotics and anticancer drugs.16−18 Bacterial DNA gyrase and topoisomerase IV are the targets of quinolones, one of the most important and prescribed antibiotics.19−22 Human topoisomerases I and II are the targets of anticancer drugs, such as camptothecin/topotecan,16,23 doxorubicin,16 and etoposide.24

Agarose gel electrophoresis is the most commonly used biochemical assay to study DNA supercoiling and topoisomerases.25−27 This assay has several advantages: it is cheap, quick, convenient, and widely accessible.1 Single topoisomer resolution can be achieved.25,26 The (−) and (+) supercoils can also be differentiated.1,28 Nevertheless, agarose gel electrophoresis is a time-consuming and labor-intensive assay and cannot be used as a high throughput screening (HTS) assay to identify topoisomerase inhibitors.27 Utilizing certain properties of sc DNA molecules, different screening assays have been established to identify inhibitors targeting DNA topoisomerases.29−33 However, these methods have their own limits. For instance, a type of unique fluorescently labeled DNA molecules has been synthesized to study DNA topoisomerases by fluorescence resonance energy transfer or supercoiling-dependent fluorescence quenching (SDFQ).33,34 This assay stems from a property of alternating (AT)n sequences in the closed circular plasmids that undergo rapid cruciform formation–deformation depending on the supercoiling status of the plasmids.35,36 The distance between a pair of fluorophore–quencher inserted in the (AT)n sequence is dramatically changed when the plasmids adopt an sc or relaxed (rx) form.33 These DNA molecules are excellent tools to...
examine relaxation/supercoiling kinetics of various DNA topoisomerases and can be configured into HTS assays to identify topoisomerase inhibitors. Indeed, we are using the SDFQ-based HTS assays to identify inhibitors targeting bacterial DNA gyrase and bacterial DNA topoisomerase I. Nevertheless, the synthesis of this type of fluorescently labeled DNA molecules is expensive. Certain potential topoisomerase inhibitors have fluorescence that greatly interferes with the final detection signal. Additionally, DNA intercalators that transiently unwind and relax the sc DNA also interfere with the identification of topoisomerase inhibitors. A different, less expensive HTS assay is needed and may be used as an orthogonal assay for the HTS efforts.

Here, we report the establishment of new types of HTS assays to discover/identify topoisomerase inhibitors based on a unique property of T5 exonuclease. Under certain experimental conditions, T5 exonuclease can completely degrade the sc plasmid pAB1 that carries a hairpin structure. In contrast, it does not digest the rx pAB1. This unique property can be used to differentiate sc and rx plasmids and then identify topoisomerase inhibitors. A 50-compound library that contains several known DNA gyrase inhibitors is used to validate these HTS assays for various DNA topoisomerases. This T5 exonuclease-based assay can also be used to identify DNA intercalators and remove them as false positives from the potential DNA topoisomerase inhibitors. Furthermore, we found a new compound that potently inhibits Escherichia coli and human DNA topoisomerase I.

**RESULTS AND DISCUSSION**

**T5 Exonuclease Completely Degraded (−) sc Plasmid pAB1 That Carries an AT Hairpin Structure.** Previous studies showed that T5 exonuclease can completely digest single-stranded DNA, linear double-stranded DNA, and nicked plasmid DNA molecules. It does not degrade (−) sc or rx plasmid DNA molecules, such as (−) sc or rx pUC18. We confirmed these results showing that the purified T5 exonuclease in our laboratory also completely removed/digested nicked (nk) pUC18 (compare lane 4 to lane 6 of Figure 1a) and did not degrade either (−) sc or rx pUC18 (lanes 5 and 6 of Figure 1a). Interestingly, we found that T5 exonuclease completely degraded (−) sc plasmid pAB1 that carries an AT hairpin structure (lane 3 of Figure 1a and Figure 2b) and, in contrast, did not digest rx pAB1 (lane 2 of Figure 1a). A likely scenario is that the endonucleolytic activity of T5 exonuclease nicked the AT hairpin structure of (−) sc pAB1 followed by the degradation of the nicked plasmid by the 5′-3′ exonuclease activity of T5 exonuclease.

The endonucleolytic activity of T5 exonuclease, i.e., the flap endonuclease (FEN) of T5 exonuclease, has been extensively studied using oligomers of the DNA flap structures, such as HP1. Specifically, T5 exonuclease cleaves the 29 nt. HP1 into two fragments, an 8 nt. fragment and a 21 nt. hairpin. In this study, we utilized a double fluorescently labeled HP1 with fluorescein (F) and TAMRA (T) on the 5′- and 3′-end (Figure 2b), respectively, to examine whether high concentrations of NTPs or dNTPs inhibit the FEN activities. Indeed, high

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concentrations of NTPs or dNTPs significantly inhibited the FEN activities of T5 exonuclease (Figure 2c,d). These results are consistent with previously published results that ATP inhibited the FEN activities of other nucleases.46,47

A T5 Exonuclease-Based HTS Assay for E. coli DNA Gyrase. Since T5 exonuclease completely degrades (−) sc plasmid pAB1 and does not digest (+) sc pAB1, this unique property of T5 exonuclease can be used to study DNA topology and topoisomerases. For instance, a T5 exonuclease-based assay may be established to identify DNA topoisomerase inhibitors. Figure 3a shows an experimental strategy of such an assay to screen/identify inhibitors targeting bacterial DNA gyrase. This assay can be easily configured into a high throughput screening (HTS) format. In the absence of gyrase inhibitors, DNA gyrase converts the (+) sc plasmid DNA templates into the (−) sc form. This conversion results in the formation of a hairpin structure in the plasmid. As a result, the (−) sc plasmid DNA template cannot be digested by T5 exonuclease (lane 5 of Figure 3b), while the (+) sc plasmid DNA template can be digested by T5 exonuclease (lane 6 of Figure 3b). DNA staining dye ethidium homodimer 1 (EthD1) was used to differentiate the DNA samples in (b). The fluorescence intensity was measured using a plate reader with excitation and emission wavelengths of 528 nm and 617 nm, respectively. Lane # are the same as (b). The symbol of “bk” represents the background fluorescence of an empty well.

A series of experiments were performed to determine the optimal conditions for the T5 exonuclease-based HTS assay for E. coli DNA gyrase (Figure S1a−c). These experiments show that 10 μM (bp) pAB1 (Figure S1a), 200 nM T5 exonuclease (Figure S1b), and 20 nM E. coli DNA gyrase (Figure S1c) are optimal and were chosen for the HTS assay. The assay tolerated up to 2% DMSO without any significant change in signal. We also found that 2 h of incubation with T5 exonuclease is needed for the assay (Figure S1d,e). Additionally, several different DNA staining dyes, i.e., Hoechst 33258, SYBR green, SYBR gold, ethidium bromide, and EthD1, with different fluorescence excitation and emission wavelengths were examined (Figure S2). All can be used in the assay depending on the fluorescence interference that the potential gyrase inhibitors have. We found that most fluorescence interference is between 400 and 500 nm. Hoechst 33258 with the maximum fluorescence emission at 461 nm should not be used although it gives the highest signal to background ratio (S/B) (Figure S2a). We chose the use of EthD1 for the HTS assays because this DNA-binding dye tightly binds to DNA and has good fluorescence signals upon DNA binding. If the budget is a concern, ethidium bromide may be used (Figure S2d). After we optimized the conditions for the T5 exonuclease-based assay for E. coli DNA gyrase, we performed titration experiments in which different concentrations of novobiocin and ciprofloxacin were added into the assays. Figure S3 shows our results. Ciprofloxacin and novobiocin potently inhibited the activities of DNA gyrase with estimated IC50 values of 3.2 and 0.48 μM, respectively. These values are consistent with our previously published results using SDFQ- and agarose gel-based assays.33

Next, we assembled a 50-compound library that contains eight known bacterial DNA gyrase inhibitors (Table S1) in order to establish and validate the T5 exonuclease-based HTS assay for E. coli DNA gyrase. Figure 4 and Table S2 show the results of this pilot screen using chemical compounds at a final concentration of 20 μM in triplicate with the following statistics: Z′, 0.64; S/B (the mean signal to mean background ratio), 3.0; and nine hits. These nine hits are novobiocin, five fluoroquinolones, suramin, echinomycin, and ethacridine. Novobiocin and five fluoroquinolones are potent gyrase inhibitors and are expected to completely inhibit E. coli DNA gyrase.
DNA gyrase activities at 20 μM. Suramin is a known DNA topoisomerase II inhibitor. It is not surprising that 20 μM suramin partially inhibits *E. coli* DNA gyrase activities. Echinomycin and ethacridine are two known DNA intercalators and should be able to significantly unwind the plasmid pAB1 at 20 μM. In this case, plasmid pAB1 was fully relaxed or (+) supercoiled. The 42 nt AT DNA sequence adopted the double-stranded form and did not form a hairpin structure. As a result, T5 exonuclease did not degrade the pAB1 DNA samples. They are false positives. Another two gyrase inhibitors nalidixic acid and oxolinic acid have IC50 values much greater than 20 μM and was not expected to inhibit *E. coli* DNA gyrase activities under the experimental conditions used here. Therefore, the T5 exonuclease-based assay could not identify these two gyrase inhibitors. Although fluoroquinolones are known to cause double-stranded DNA breaks through stabilizing DNA-gyrase-drug complexes, we were still able to identify them as potent gyrase inhibitors using this HTS assay. A possible mechanism is that DNA gyrase covalently links to the plasmid DNA substrate when fluoroquinolones stabilize the DNA-gyrase-fluoroquinolone complexes, which prevents the access of T5 exonuclease to the DNA breaks. As a result, DNA gyrase was inhibited and the DNA substrate (rx pAB1) was not digested.

**A T5 Exonuclease-Based HTS Assay to Identify Potential DNA Intercalators and T5 Exonuclease Inhibitors.** As stated above, DNA intercalators can unwind and transiently relax the (−) sc plasmid pAB1. High concentrations of DNA intercalators may even convert (−) sc plasmids into (+) sc plasmids. Combining with the unique property of T5 exonuclease that completely degrades (−) sc plasmid pAB1 and does not digest rx pAB1, a T5 exonuclease-based assay may be used to identify potential DNA intercalators, the major false positives for the HTS assays. Figure 5a shows the experimental strategy for this purpose. DNA intercalators can convert (−) sc pAB1 into the rx form. As a result, the 42 nt AT DNA sequence adopts the double-stranded form and T5 exonuclease cannot degrade this transiently rx pAB1. In contrast, T5 exonuclease is able to completely digest (−) sc pAB1. As discussed above, a DNA-binding dye should differentiate these two forms of DNA samples. Please note that the positive hits also include potential T5 exonuclease inhibitors since the inhibition of T5 exonuclease also prevents the degradation of (−) sc pAB1 and results in the high fluorescence output.

We screened the 50-compound library using this assay at a final compound concentration of 20 μM in triplicate and got the following statistics: Z’ = 0.63; S/B = 5.5; and three hits (suramin, echinomycin, and ethacridine) (Figure 5b). Echinomycin and ethacridine are potent DNA intercalators with a Keq (dissociation constant) at the sub-micromolar range and form strong DNA intercalation complexes. It is expected that both echinomycin and ethacridine fully relax (−) sc plasmid pAB1 at 20 μM. As a result, T5 exonuclease could not degrade the rx pAB1 and yielded a high fluorescence output (Figure 5b). Suramin, a century-old antiparasitic drug that carries 6 negative charges under physiological conditions, inhibits many enzymes including those involved in nucleic acid metabolisms. Possibly, suramin also inhibits T5 exonuclease. Indeed, our results showed that suramin inhibits the endonucleolytic or FEN activity of T5 exonuclease with an IC50 of 22.6 μM (Figure S4a–c).

**T5 Exonuclease-Based HTS Assays for DNA Topoisomerases That Relax (−) sc Plasmid DNA Molecules.** We also established several T5 exonuclease-based HTS assays to screen and identify inhibitors for DNA topoisomerases, e.g., bacterial DNA topoisomerase I, human DNA topoisomerase I, and human DNA topoisomerase IIa that relax (−) sc DNA templates. Figure 6a shows an experimental strategy to screen/identify inhibitors targeting *E. coli* DNA topoisomerase I (EcTopI). In the absence of an inhibitor, EcTopI relaxes the sc plasmid pAB1 into the rx form. This relaxation results in the conversion of the hairpin structure of pAB1 into the double-stranded form. In this case, T5 exonuclease cannot digest the rx pAB1 (lane 2 of Figure 1a). In contrast, an EcTopI inhibitor prevents the conversion of the sc plasmid pAB1 into the rx form. T5 exonuclease completely degrades sc pAB1. A DNA staining dye, such as ethidium homodimer 1 (EthD1), can be used to differentiate these two DNA samples.

We screened the 50-compound library using this assay and found three hits. The following are the screening statistics using a compound concentration of 20 μM: a Z’ value of 0.68 and S/B ratio of 3.3. These three hits are suramin, NSC668394, and NSC97419. As mentioned above, the highly negatively charged suramin inhibits many enzymes. It is not surprising that suramin also inhibits EcTopI. Indeed, our agarose gel-based topoisomerase assays confirmed this result (Figure S4d). We could not confirm that NSC97419, a polyphenol, inhibits EcTopI activities using the agarose gel-
based topoisomerase assay. The most intriguing compound is the quinolinedione NSC668394, an ezrin inhibitor53,54 that almost completely inhibited the EcTopI activities in the HTS assay (Figure 6b and Figure S5). We, therefore, performed an agarose gel-based EcTopI inhibition assay and found that NSC668394 strongly inhibited the EcTopI activities with an IC50 of 8.0 ± 0.2 μM (Figure 7a). Since NSC668394 carries a benzoquinone moiety that should be reactive toward thiol groups,55 a possible mechanism of this inhibition is through the formation of a covalent bond with cysteine residues of EcTopI since certain cysteine residues are critical for the EcTopI activities.56 If this hypothesis is correct, DTT should be able to abolish the EcTopI inhibition of NSC668394.

Figure 7b shows our results. Indeed, NSC668394 did not inhibit EcTopI activities in the presence of 1 mM DTT (compare lane 1 with lanes 2 and 3 of Figure 7b). The visible spectra of NSC668394 in the presence and absence of DTT are dramatically different, indicating that NSC668394 formed covalent bonds with thiols of DTT (Figure 7c).

Human DNA topoisomerase I (HuTopI) also contains eight cysteine residues that are critical for its activities.57 Previous results showed that alkylation of the HuTopI cysteine residues by thiol modification compounds, such as N-ethylmaleimide, resulted in the loss of HuTopI activities in vitro.57,58 We wondered whether NSC668394 could also inhibit the HuTopI activities. Our results in Figure 7d show that NSC668394 strongly inhibited HuTopI with an IC50 of 5 ± 1.4 μM. Again, DTT abolished the inhibition of NSC668394 against HuTopI (Figure S4e), suggesting that the inhibition is through the formation of covalent bonds with the thiol groups of cysteine residues in the enzyme.

In this study, we also developed a T5 exonuclease-based HTS assay (Figure S6a) to identify inhibitors targeting variola DNA topoisomerase I (vTopI), a type IB DNA topoisomerase, and screened the 50-compound library with the following statistics: Z’ 0.66; S/B ratio, 5.8; and one hit. The only positive hit is the polynegatively charged suramin (Figure S6b).

■ CONCLUSIONS

In this article, we discovered that T5 exonuclease completely degraded (−) sc plasmid pAB1 that carries an AT hairpin structure. In contrast, T5 exonuclease did not digest rx plasmid
pAB1. Using this unique property of T5 exonuclease, we developed a series of HTS assays that could be used to identify inhibitors targeting various DNA topoisomerases. We also developed an HTS assay to identify inhibitors for T5 exonucleases and potential DNA intercalators. We validated these HTS assays using a 50-compound library. All HTS assays have a Z' more than 0.5 and are ready to screen different compound libraries. Additionally, we found that compound NSC668394, an ezrin inhibitor potently inhibits both EcTopI and HuTopI. It is likely that NSC668394 inhibits EcTopI and NSC668394, an ezrin inhibitor potently inhibit both EcTopI and HuTopI through the formation of covalent bonds with the thiol groups of cysteine residues of these two enzymes. An HuTopI inhibition of NSC668394 (EthD1) or another DNA-binding dye was added to each well, the fluorescence was measured in a microplate reader. For EthD1, fluorescence was measured at λ_em = 617 nm with λ_exc = 528 nm.

The Z-factor (Z') was determined using the 96 wells of a 384-well plate where 48 wells are for positive controls in the presence of 3 μM novobiocin and the remaining 48 wells for negative controls in the absence of novobiocin. Z' was calculated by the following equation:

\[ Z' = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \]

where σ_p, σ_n, μ_p, and μ_n represent the sample means and standard deviations for positive (p) and negative (n) controls, respectively.

**T5 Exonuclease-Based Assays to Identify Inhibitors for E. coli and Variola DNA Topoisomerase I.** All assays were conducted in 384-well plates using 1× CutSmart buffer (20 mM Tris-acetate pH 7.9, 50 mM KAc, 10 mM Mg(AC)_{2}, and 100 μg/mL BSA). Briefly, 20 nM E. coli or variola DNA topoisomerase I (nM) was used to relax the (−) sc pAB1 (200 ng; 10 μM (bp)) at 37 °C for 30 min in the presence of a potential inhibitor. The E. coli or variola DNA topoisomerase I was incubated with a potential inhibitor at 37 °C for 5 min before the sc pAB1 was added into the reaction mixtures. Next, 200 nM T5 exonuclease was added into the reaction mixtures. The 384-well plate was incubated at 37 °C for additional 120 min. After ethidium homodimer 1 (EthD1) was added to each well, the fluorescence was measured in a microplate reader.

**T5 Exonuclease-Based Assays to Identify DNA Intercalators.** These assays are identical to the T5 exonuclease-based assays for E. coli and variola DNA topoisomerase I except E. coli, and variola DNA topoisomerase I are omitted. Briefly, 30 μL of 1× CutSmart buffer containing 200 ng of (−) sc pAB1 in the presence of a potential DNA intercalator is used. Next, 200 nM T5 exonuclease was added into the reaction mixture. The 384-well plate was incubated at 37 °C for additional 120 min. After ethidium homodimer 1 (EthD1) was added to each well, the fluorescence was measured in a microplate reader.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00962.

Determining the optimal conditions for the T5 exonuclease-based HTS assay to screen and identify E. coli DNA gyrase inhibitors; different DNA staining dyes were used in the T5 exonuclease-based DNA topoisomerase assays; DNA gyrase was potently inhibited by ciprofloxacin and novobiocin; suramin inhibits E. coli DNA topoisomerase I activities; DTT abolishes the HuTopI inhibition of NSC668394; identity of NSC668394 was confirmed by ESI-MS; TSE-based experimental strategy to identify inhibitors targeting variola DNA topoisomerase I (vTopI); HTS pilot screen...
of the 50-compound library for vTopI inhibitors; and parameters for the TS exonuclease-based HTS assay for E. coli DNA gyrase using the 50-compound library (PDF)

Chemical structures and vendors of the 50 compounds (XLSX)

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Author Contributions
F.L. and Z.D. designed research; Z.D. performed research; F.L. and Z.D. analyzed data; and F.L. wrote the paper.

Notes
The authors declare the following competing financial interest(s): A provisional patent application has been filed for the TS exonuclease-based assays.

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