Single-molecule Förster resonance energy transfer (FRET) analysis discloses the dynamics of the DNA–topoisomerase II (Top2) interaction in the presence of TOP2-targeting agents

Wan-Chen Huang1, Chun-Ying Lee2,* and Tao-shih Hsieh†§¶†‡

From the 1Institute of Cellular and Organismic Biology, Academia Sinica, Taipei 115, Taiwan, the 2Department of Chemistry, National Taiwan University, Taipei 106, Taiwan, and the 3Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Edited by Patrick Sung

Topoisomerases play crucial roles in DNA replication, transcription, and recombination. For instance, topoisomerase II (Top2) is critically important for resolving DNA tangles during cell division, and as such, it is a broad anticancer drug target. Top2 regulates DNA topology by transiently breaking one double-stranded DNA molecule (cleavage), allowing a second double-strand to pass through the opened DNA gate (opening), and then closing the gate by rejoining the broken ends. Drugs that modulate Top2 catalysis may therefore affect enzymatic activity at several different steps. Previous studies have focused on examining DNA cleavage and ligation; however, the dynamic opening and closing of the DNA gate has been less explored. Here, we used the single-molecule Förster resonance energy transfer (smFRET) method to observe the open and closed state of the DNA gate and to measure dwell times in each state. Our results show that Top2 binds and destabilizes DNA to increase the energy transfer efficiency (\(E_{\text{FRET}}\)), and ATP treatment further induces the fluctuation of \(E_{\text{FRET}}\) representing the gate opening and closing. Additionally, our results demonstrate that both types of Top2-targeting anticancer drugs, the catalytic inhibitor dexrazoxane (ICRF187) and mechanistic poison teniposide (VM26), can interfere with DNA gate dynamics and shorten the dwell time in the closed state. Moreover, Top2 bound to the nonhydrolyzable ATP analog ATPγS-exhibits altered DNA gate dynamics, but the DNA gate appears to open and close even after N-gate closure. In summary, we have utilized single-molecule detection to unravel Top2 DNA gate dynamics and reveal previously unknown effects of Top2 drugs on these dynamics.

Topoisomerases play crucial roles in DNA replication, transcription, and recombination (1–4). Frequent unwinding and rewinding of the DNA duplex during these processes can create topological obstructions to DNA transactions, and failure to properly resolve these obstructions can induce cell death (4–6). Topoisomerases resolve topological strain by breaking a DNA strand and allowing a second strand to pass through before rejoining the broken ends (7). Depending on the substrate specificity, topoisomerases can be categorized into two types, types I and II, which process single-stranded and double-stranded DNA, respectively. Whereas type I topoisomerases are specifically involved in transcription, recombination, and DNA repair, type II topoisomerases are required for DNA replication and segregation of catenated chromosomes (5, 7). Because cancer cell growth is heavily dependent on type II topoisomerase functions, they have become critical targets for anticancer drugs (4, 8).

The catalytic cycle of topoisomerase II (Top2) obeys a two-gate mechanism and requires both ATP and Mg2+ to trigger the strand passage reaction (3, 9). The cycle is initiated when the core domains of a Top2 homodimer bind to a DNA fragment (G-segment or G-DNA), which is then bent to a 150° angle (10). The N-gate will close and trap the T-segment upon ATP binding to the ATPase domain. Concurrently, the tyrosine residues inside the core domain participate in a transesterification reaction and form covalent adducts with the DNA backbone, breaking the G-segment (cleavage) and pulling the broken ends apart (opening) to produce a double-stranded DNA break with 4-bp overhangs. This conformation is referred to as the open DNA gate. The hydrolysis of one ATP is accompanied by the passage of the T-segment through the opened gate (11). Next, the DNA gate is closed, bringing the ends of the G-segment into close proximity for rejoining (ligation) via the second transsterification reaction. Finally, a second ATP hydrolysis dissociates the closed N-gate, which allows the release of the G-segment from Top2 (12). Thus, the opening and closure of the DNA gate are closely related to, but functionally distinct from cleavage and ligation reactions.

The Top2 complex with cleaved DNA can be stabilized by the anticancer drugs, etoposide (VP16) and teniposide (VM26), which interfere with DNA ligation. This action results in accumulation of DNA breaks that are poisonous to cells, and the drugs are therefore referred to as DNA poisons (11, 13). Moreover, two bisdioxopiperazine-type antitumor agents, ICRF193 and ICRF187 (dexrazoxane), which are termed catalytic inhibitors, block ATPase activity and promote the closed clamp formation, resulting in impaired enzyme turnover and T-segment...
Single-molecule study of topoisomerase 2 gate dynamics

passage (14). Although biochemical studies on Top2-targeting drugs have elucidated many features of Top2 inhibition, a complete delineation of the mechanism, in terms of the interference with gate dynamics, remains to be uncovered. For example, whether Top2 drugs alter DNA gate dynamics and how this alteration may correlate with N-gate activity to perturb Top2 function is previously unknown.

Currently, several high-resolution fluorescence techniques are available for the precise physicochemical characterization of elemental processes at the level of a single protein or nucleic acid. Similar FRET methods have been used to monitor the DNA gate recently (15–18). However, previous studies did not address the ability of Top2 drugs to interfere with DNA gate dynamics. Here, we used single-molecule Förster resonance energy transfer (smFRET),2 a technique based on total internal reflection fluorescence (TIRF) microscopy, to observe the DNA gate dynamics of Top2. Dynamic measurements were made in the presence of cofactors, ATP and AMPPNP, as well as anticancer drugs ICRF187 and VM26. Based on our experiments, we report that (i) DNA gate dynamics are altered by treatment with either Top2 poisons or catalytic inhibitors, which both shorten the gate closed time, and (ii) DNA gate opening and closing may persist after N-gate closure.

Results

smFRET detection of DNA opening and closing

To observe the dynamics of the DNA gate as it goes through different conformational states during the catalytic cycle, we performed TIRF microscopy-based smFRET measurements. The DNA substrate was designed such that the fluorophore adducts were located 2 bp outside the DNA footprint of Top2 (Fig. 1B) (15). The DNA sequence used for smFRET experiments had an additional biotinylated 23-nt poly(T) sequence at the 5′-end of the same strand with AF488, which allowed the DNA molecule to be immobilized on the coverglass surface. The FRET measurement was performed after reaction buffer was infused into the reaction chamber. The distribution of FRET efficiency and the dynamics of single molecules were collected in the absence or presence of DmTop2, ATP, and VM26. Five separate recordings of single molecules were made in randomly selected areas, and the average FRET efficiency between the first 10 and 20 frames was taken as the average FRET value. The FRET-distribution histograms were then fitted with a double-Gaussian peak-fit function (Fig. 2A). When only DNA was present (top panel), the FRET peak was centered at \( E = 0.36 \), indicating the FRET value for the native B-DNA conformation. After 300 nm DmTop2 was infused (second panel), the FRET value shifted to \( E_1 = 0.49 \). The increased FRET value is consistent with bending of the DNA duplex, which should reduce the distance between the two fluorophores and increase the energy-transfer efficiency. Intriguingly, a small population of molecules exhibited a lower FRET peak (at \( E_2 = 0.29 \)), and after ATP addition, this population increased from 10 to 17% (third panel). The opening of the DNA gate would create a longer distance between the fluorophores and thus a lower FRET efficiency. Moreover, the fraction of molecules in the lower peak increased to 23% when 200 \( \mu \)M teniposide (VM26, which prevents DNA ligation) was added to the observation chamber (bottom panel). This observation may suggest that by keeping the DNA backbone unlocked, there is a greater chance for the opened DNA gate to be observed.

Mg\(^{2+}\) is thought to be essential for the DNA cleavage and ligation steps (19). Therefore, to determine whether the lower FRET peak (Fig. 2A) was caused by DNA cleavage-opening after ATP addition, we obtained a parallel set of measurements using reaction buffer without Mg\(^{2+}\) (Fig. 2B). When 300 nm DmTop2 was added to the reaction chamber, the FRET value increased from 0.34 to 0.47, which suggests that DNA binding and bending by DmTop2 occurred in the absence of Mg\(^{2+}\). However, the lower FRET peak \( (E_{\text{FRET}} = 0.29) \) was not detected, further supporting the notion that the FRET value at 0.29 represents the cleavage-opening of the DNA gate. When the unlocking of the DNA backbone is inhibited (by removing Mg\(^{2+}\)), the opened state cannot be achieved.

We further analyzed each single-molecule time trace obtained from the treatment with DmTop2, ATP, and/or VM26. We aimed to determine whether we could observe the transient/intermediate states with the smFRET method. Therefore, we used the hidden Markov model (HMM) to fit all of the FRET trajectories with the maximum of five states using vbFRET in a Matlab environment. No assumptions were made about the values or distributions. Hundreds of trajectories were fitted to generate each distribution, and from the fitted FRET results, we found the trajectories with transitions are almost entirely between two states. According to the \( E_{\text{FRET}} \) and \( \Delta E_{\text{FRET}} \) values for each FRET trajectory, we were able to classify the possible types of transitions. The representative FRET trajectory in Fig. 2C show multiple transitions between high and low FRET and variable dwell times in each state. The distribution of dwell times calculated from the fitted FRET values is plotted in Fig. 2D. High FRET panels show the distribution of dwell times at the higher FRET value \( (E_1) \), 0.5, which represents bending of DNA. Low FRET panels show the distribution of dwell times at the lower FRET value \( (E_2) \), 0.29, which is taken to represent the open state of the DNA gate. Rate constants were determined by fitting a single exponential function to the dwell time histograms, and the average dwell time was calculated. Intriguingly, in the Top2-treatment group without ATP, FRET transitions were detected in some of the single molecules (Fig. 2C, upper panel) and exhibited a relatively long dwell time, \(~2.3\) s, in the closed-gate state and a short opening time of \(~0.2\) s (Fig. 2D), however, the FRET value of most molecules (more than 90%) remained constant throughout the observation period (supplemental Fig. S1). With ATP treatment, the dwell time at high FRET was shortened to \(~1.2\) s and the low-FRET dwell time was \(~0.4\) s. In the VM26-treatment group, we observed single molecules displayed either one of two possible transition patterns. In the slow-transition group, the dwell time was similar to that of the ATP-treatment group (supplemental Fig. S2). However, in the fast-transition group, the high-FRET dwell time was shortened to \(~0.5\) s, and the low-FRET dwell time was slightly reduced to \(~0.3\) s (Fig. 2D, bottom panel). This result

---

2 The abbreviations used are: smFRET, single-molecule Förster resonance energy transfer; TIRF, total internal reflection fluorescence; AMPPNP, 5′-adenyl-β,γ-imidodiphosphate; HMM, hidden Markov model.
suggested that Top2 tends to open the DNA gate more readily in the presence of VM26 than in its absence.

**Interference effect of AMPPNP and Top2 drugs on DNA gate open and closed states**

AMPPNP, a nonhydrolyzable analog of ATP, has been reported to bind to the ATP-binding pocket of Top2 and allow a single turnover of T-DNA transport through the Top2 DNA gate (20, 21). Thus, we sought to examine DNA gate dynamics after AMPPNP binding. Fig. 3A shows the FRET-distribution histograms of the DNA substrate alone, with DmTop2, and with DmTop2 plus AMPPNP. We observed that the low-FRET population was slightly increased in the AMPPNP-treatment group. Upon further analyzing the single-molecule FRET trajectories (Fig. 3B), we observed frequent gate opening and closing events indicated by the transitions between two FRET efficiency levels. Fig. 3C shows that after AMPPNP treatment, the dwell times in the high-FRET and low-FRET states were similar, 0.57 and 0.43 s, respectively. Observing FRET transitions in single molecules with AMPPNP treatment was unexpected, and we initially considered these findings may be contrary to previous reports, which have generally supported the view that AMPPNP induces N-gate closure and the trapping of the DNA gate (22, 23). It was previously reported that DNA cleavage can occur in the presence of AMPPNP (24). Therefore, we performed a gel-based DNA-cleavage assay to test whether DNA cleavage occurs in our experimental system. The gel results (Fig. 3D) showed that whereas ATP induced the relaxation of supercoiled DNA into open-circular form, AMPPNP did not. Furthermore, AMPPNP induced a faint band corresponding to the linear-form DNA. VM26, a Top2 poison, impedes Top2 ligation and thereby generates DNA break. When we applied
Single-molecule study of topoisomerase 2 gate dynamics

![Graphs showing FRET efficiency and relative counts for DmTop2, ATP, ATP+VM26 with and without Mg²⁺]

C

DmTop2

![Graphs showing IAU and FRET efficiency over time for DmTop2, ATP, and ATP+VM26]

D

![Graphs showing decay time constants τ_close and τ_open for DmTop2, ATP, and ATP+VM26]

J. Biol. Chem. (2017) 292(30) 12589 – 12598
**Figure 2.** *DmTop2* induces DNA bending, as well as DNA gate opening and closing. A, FRET efficiency-distribution histograms of the interaction between *DmTop2* and DNA. The distribution of FRET efficiency values ($E_{\text{FRET}}$) was determined from hundreds of single molecules in the absence or presence of *DmTop2*, ATP, and VM26. B, Mg$^{2+}$ was removed from the reaction buffer and similar experiments were performed in parallel with A. C, representative intensities and corresponding FRET trajectories from single-molecule measurements. D, the histogram of dwell time at each state (gate closed dwell time, $\tau_{\text{close}}$; gate open dwell $\tau_{\text{open}}$) was fitted with a single exponential function.

**Figure 3.** AMPPNP and Top2 poisons interfere with DNA gate dynamics. A, FRET-distribution histograms of DNA alone and *DmTop2* with or without AMPPNP. B, representative time and FRET trajectories after AMPPNP treatment. C, dwell time distribution of the high- and low-FRET states after AMPPNP treatment. Rate constants were determined by fitting an exponential decay function to the histogram derived from a normalized population of dwell times. D, results of a DNA-cleavage assay showing increased relaxed circular DNA (RC) by *DmTop2* with ATP but not with AMPPNP. In the presence of VM26 both ATP and AMPPNP treatments showed increased linear DNA. Lanes are labeled as follows: D, DNA; L, linearized DNA; M, AMPPNP; T, ATP, with and without VM26. NC, nicked circular DNA; LC, relaxed circular DNA; E, FRET-distribution histogram showing that the low-FRET population was increased when gate ligation was impaired by VM26.
Single-molecule study of topoisomerase 2 gate dynamics

VM26 to both the ATP- and AMPPNP-treated groups, a considerable amount of linearized DNA was observed in the presence of ATP, indicating DNA cleavage. However, an increase in linear DNA also appeared in the AMPPNP plus VM26 co-treatment group. This result indicated that DNA gate opening and closing could still occur after AMPPNP treatment, and that VM26 interfered with subsequent ligation steps resulting in the increased generation of linear DNA. The FRET-distribution histogram revealed that the low-FRET population was further increased after AMPPNP treatment when gate ligation was hindered by VM26 (Fig. 3E).

Impairment of DNA gate opening and closing by Top2 catalytic inhibitors

Previous biochemical data have provided evidence that two Top2 catalytic inhibitors, ICRF193 and ICRF187, can reduce Top2-mediated DNA-relaxation activity and promote the closed-clamp formation of the protein (14). However, whether these inhibitors interfere with DNA gate opening and closing is unclear. Thus, we performed smFRET measurements with various concentrations of Top2 catalytic inhibitors (Fig. 4). Whereas the dwell time at the low-FRET state increased when the ICRF187 concentration was raised from 200 to 400 nM, the dwell time at the high-FRET state remained unchanged. Similarly, in the ICRF193-treatment group, the dwell time was longer in the low-FRET state than at the high-FRET state (Fig. 4B). We fitted the histogram of low FRET dwell time distribution in Fig. 4B with a double exponential decay function. This could better fit the dwell times, suggesting the possibility that two components exist: one component is transient, at around 0.25–0.3 s, and one is longer, at around 1.3–1.6 s, gate opening time (data not shown). However, fitting with multiexponential decay functions can always produce better fitted results, and might generate false states. Therefore, we conclude that fitting with single exponential decay function is preferred. These results suggest that Top2 catalytic inhibitors might slightly prolong the open state of the DNA gate.

Discussion

We used the smFRET system to study the dynamics of Top2 interaction with DNA at different steps of the catalytic cycle, including DNA bending, DNA gate opening, and DNA gate closing. With this methodology, we determined the effect of ions, cofactors, and Top2 anticancer drugs on protein dynamics. Our data demonstrate the utility of this novel tool for studying the effects of Top2 drugs on the conformational dynamics of the enzyme. The use of smFRET complements previously established methods in probing the mechanisms by which drugs might exert their effects on Top2.

When DNA alone was present, the FRET efficiency peak was centered at 0.36. After a saturating amount of DmTop2 was introduced into the reaction chamber, our data suggest the binding and bending of the DNA duplex brought the two fluorophores closer together, resulting in an increase in the energy-transfer efficiency, whereas a small population of molecules exhibited a lower FRET peak (at 0.29) meaning that adding Top2 alone can initiate opening of the DNA gate. Moreover, with ATP, the FRET efficiency decreased in a subpopulation of molecules, representing the state when the DNA gate is open. Mg²⁺ can unlock the DNA gate and form a triad with conserved acidic amino acid residues that are situated in the core domain (13, 25). In the group treated with only Top2, a small proportion of the single molecules showed FRET transitions with a short open gate dwell time of ~0.2 s, but the DNA gate of most of the single molecules was closed throughout the entire observation period. When Mg²⁺ was removed from the reaction buffer (Fig. 2B), DNA bending was not affected, but DNA gate opening could no longer occur. Suggesting the essential role of Mg²⁺ for the Top2-induced DNA gate opening.

According to our control experiments with DNA only and Top2 plus DNA, we found that these two states have close but distinct $E_{\text{FRET}}$ values of 0.36 and 0.5, respectively. Therefore, in our experimental design, we are able to tell the difference between Top2 dissociation from DNA and Top2 binding-induced DNA bending, according to the $\Delta E_{\text{FRET}}$ values and FRET trajectories. Within our smFRET observation period, which is around 20 s on average, we readily observed FRET fluctuations between 0.5 and 0.29 (with $\Delta E$ ranging from 0.18 to 0.25). Only a few trajectories showed FRET transitions between 0.36 and 0.5 (with $\Delta E \approx 0.1$–0.15), but we do not observe the combination of FRET transitions among these three states. Therefore, we are confident that we were able to extract properly the dwell time information. However, we are not able to exclude the possibility that random association of Top2 and DNA occurs. In this case, FRET values would not change with bending, and the DNA cleavage-related opening would also not be observed.

ATP is a cofactor that can promotes T-segment capture and stimulates G-segment cleavage (9, 22, 26–28). Pre-steady-state analysis of ATP hydrolysis using Saccharomyces cerevisiae (Sc) Top2 and DNA has previously indicated that the enzyme binds and hydrolyzes two ATP molecules per reaction cycle, with the hydrolysis of the ATP molecules occurring sequentially (27, 29). In the smFRET data obtained here, we did not observe any consistent pause between the two cycles of DNA gate opening and closing, but instead observed a continuous opening and closing in the presence of ATP. This result suggests that ATP is continuously hydrolyzed to open and close the DNA gate, with an average dwell time in the open and closed states that can be modified by the presence of Top2-targeting drugs. The sum of dwell times in the high- and low-FRET states can describe the completion of a single cycle of DNA opening and closing. Without T-segment, the average time for a full cycle can be estimated from the DmTop2 plus ATP condition, and is ~1.54 s.

Top2-targeting drugs are divided into two classes, based on their mode of action. One class is Top2 poisons, such as VM26 and VP16, which result in DNA double-strand breaks. The other class, which includes inhibitors such as ICRF193 and ICRF187, suppresses ATPase activity. In the presence of ATP, the reaction equilibrium strongly favors ligation over cleavage, and thus at any given time, only a small fraction of the enzyme is covalently attached to cleaved DNA (30). However, when VP16 impedes the ligation at the DNA gate, a comparatively larger fraction of the enzyme remains covalently attached to cleaved DNA. Our smFRET results demonstrated that the VM26-treatment group showed considerably faster opening kinetics compared with the group treated with ATP alone.
**Figure 4. Top2 catalytic inhibitors modulate DNA gate dynamics.** Dwell time distribution of high- and low-FRET states after treatment with ICRF187 (A) and ICRF193 (B) at two concentrations. The histogram of the dwell time at each state (gate closed dwell time: $\tau_{\text{close}}$, high $E_{\text{FRET}}$; gate open dwell time: $\tau_{\text{open}}$, low $E_{\text{FRET}}$) was fitted with a single exponential function.
Thus, when ATP binds to initiate a new catalytic cycle, the continued presence of a previously cleaved G-segment, due to the impairment of ligation by VM26, may allow Top2 to readily open the DNA gate again. This phenomenon might also explain why gate dynamics cannot be monitored with Top2 protein that only contains the core domain. When the ATP-binding domain is absent, even if the G-segment is cleaved, the mechanical force from N-gate closure would be absent. This force might be required to twist the protein conformation and open the DNA gate. Therefore, our data suggest that Top2 poisons, such as VM26, may prevent ligation of the G-segment, but they do not prevent ATP-dependent opening and closing of the DNA gate. These results appear to contradict the widely-held belief that VM26 and VP16 can trap Top2 as cleavage complex. Nonetheless, our results suggest a scenario where VM26 shortens the gate-closed time, allowing the DNA gate to open more frequently, but does not alter the amount of time the DNA gate remains open. We speculate that the reduced gate-closed time may be too transient for N-gate to properly trap T-DNA. Therefore, although our data suggest strand passage might still be possible in the presence of VM26, the drug may disrupt the coordination between the DNA gate and T-segment trapping by the N-gate, and thereby limit T-segment passage to some extent.

We added a saturating amount of VM26, according to the molar ratio of drug to immobilized DNA molecules, however, we still observed two transition patterns. It is possible that these different patterns represent different binding modes of VM26. For instance, the number of VM26 drug molecules bound (either one or two molecules) may differentially interfere with the ligation sites in Top2, causing different dynamics. One transition pattern exhibits dwell times that are very similar to the shut transition pattern exhibits dwell times that are very similar to the shut transition pattern, with high FRET of T<sub>close</sub> = 1.41 s and low FRET of T<sub>open</sub> = 0.49 s (supplemental Fig. S2). This may suggest the possibility that VM26 did not in fact saturate all the Top2–DNA complexes. As a result, some of the DNA gates seem to exhibit dynamics that remain unaltered by the VM26 drugs.

With regard to Top2 catalytic inhibitors, a previously unrecognized mechanism of cell killing was reported for ICRF187 and ICRF193 (31, 32). ICRF193 was discovered to be a strong Top2 poison that exhibits high potency toward Top2β. In that study, guanidine hydrochloride was used as the denaturant to terminate the reaction instead of SDS (33). The crystal structure of the ScTop2 ATPase region bound to ADPPNP and ICRF197 revealed that ICRF187 functions by bridging and stabilizing a transient dimer interface between two ATPase protomers, and thus, blocking enzyme turnover (34). However, these studies did not provide the crystal structure of the core domain or the DNA gate, and moreover, gate dynamics cannot be directly addressed with crystallization studies. Therefore, the effect of catalytic inhibitors on DNA gate dynamics has remained largely unknown. Our smFRET results, presented in Fig. 4A, show that the dwell time in the gate-open state increased as the ICRF187 concentration was increased from 200 to 400 μM. Similarly, the results in Fig. 4B show that in the ICRF193-treatment group, dwell time was longer at the low-FRET/open state than at the high-FRET/closed state. A possible explanation for this complex picture is that bisdioxopiperazines inhibit both ATP hydrolysis steps, but are not competitive inhibitors of ATP (35). Therefore, drug-bound Top2 may continue to hydrolyze ATP, but at a reduced rate. However, in our data, we could not exclude the possibility that catalytic inhibitors preferentially inhibit a single ATP hydrolysis reaction. Thus, it might still be possible that preferential inhibition of the second ATP hydrolysis might prolong the gate opening time (4). Overall, our smFRET findings might provide mechanistic insight to further explain the effects of ICRF treatment on the interaction of Top2 with DNA.

Previous findings obtained using a filter-binding assay suggested that DNA cleavage occurs after AMPPNP binding (22). Furthermore, in a biochemical gel-based assay, DNA cleavage was enhanced by treatment of AMPPNP compared with ATP (24). Crystal structures of full-length ScTop2 complexed with DNA and AMPPNP (23) revealed a double-domain-swapping event between the ATPase domains. However, no insights were obtained regarding the DNA gate dynamics induced by AMP-PNP-treated DmTop2. Biochemical assays, such as DNA-cleavage assays, may help reveal DNA locked (G-segment ligated) and unlocked (G-segment cleaved) states; however, the dynamics of gate opening and closing cannot be readily observed using traditional biochemical assays. Intriguingly, the results of our gel-based assays showed that DNA cleavage was enhanced by VM26 in the presence of AMPPNP. Although the biochemical reaction was terminated using SDS and proteinase K, linear DNA was increased in the group treated with both AMPPNP and VM26, supporting the possibility that the DNA gate may continue to open and close while the N-gate is closed. However, the energy source that drives the opening and closing of the DNA gate without ATP hydrolysis will need to be further elucidated.

In summary, by establishing and using smFRET detection of DNA gate dynamics, we have elucidated previously unidentified Top2–DNA interaction states that are induced by AMPPNP and Top2 drugs. The use of this methodology not only provides detailed mechanistic information on Top2 drugs, but also offers an approach for discovering other potential Top2 drugs that act through novel mechanisms.

**Experimental procedures**

**Protein and DNA substrate preparation**

Singly labeled single-stranded DNA substrates were purchased from Integrated DNA Technologies (Coralville, IA). The complementary nucleotide sequences and fluorophore-labeled double-stranded DNA locations are shown in Fig. 1B. After annealing, dual-fluorophore-labeled double-stranded DNA was used for FRET; singly labeled double-stranded DNA served as the control. *Drosophila melanogaster* Top2 (DmTop2) containing an N-terminal hexahistidine tag was purified using Ni<sup>2+</sup>-affinity and ion-exchange chromatography (36).

**Immobilization of DNA molecules**

Glass coverslips (#1) were cleaned with methanol, NaOH, and HCl for 10 min each with sonication and treated with 3-aminopropyltriethoxysilane (Sigma), and this was followed by passivation with methoxy-PEG (m-PEG-5000, Laysan Bio,
Inc.) and 1% biotin-PEG (biotin-PEG-5000, Laysan Bio, Inc.) to minimize nonspecific binding. After assembling the coverslips by using a 6-channel ibidi sticky slide (Sticky-slide VI 0.4, ibidi, GmbH, Germany), the chamber was filled with streptavidin solution, and the coverslips were incubated for 5 min. After washing with TEN buffer (10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 50 mM NaCl), 15 pm biotinylated DNA was surface-immobilized, and then excess DNA was removed by washing the chamber with TEN buffer.

**Single-molecule FRET measurement**

All single-molecule measurements were carried out at room temperature in the presence of 2 mM Trolox in addition to the DmTop2 reaction buffer, which contained 20 mM Tris-HCl, pH 7.9, 50 mM KCl, 0.1 mM EDTA, and 10 mM MgCl₂. Data acquisition was initiated after the reaction buffer containing DmTop2 was injected. ATP and/or Top2 drugs were subsequently added to the chamber and data were acquired. Single-molecule fluorescence studies were performed using an inverted Olympus IX81 microscope equipped with an NA1.49 UAPON ×100 oil-immersion TIRF objective. Molecules were excited with a 488-nm laser (diode laser, 60 milliwatt), and the fluorescence emission was passed through a set of optics that included a long-pass filter to reject scattered laser light (DM505lp) and dichroic mirrors to split donor and acceptor channels (ET535/30m and ET575/40m, Chroma). Movies were recorded with an iXon3 897 electron-multiplying charge-coupled device camera (Andor) at a 50-ms time resolution for 2 min, and then processed using IDL (Interactive Data Language) and a custom data-acquisition and analysis software package (Center for the Physics of Living Cells, University of Illinois at Urban-Champaign) to produce and analyze fluorescence-intensity time traces. After correction of the donor (ID) and acceptor (IA) intensities for cross-talk between the two channels, FRET efficiencies ($E_{\text{FRET}}$) were calculated as $E_{\text{FRET}} = \frac{\text{IA}}{\text{ID} + \text{IA}}$. For unbiased identification of multiple states, each time trace was then processed using vbFRET software (37), with the maximum of 5 states based on the HMM and maximum evidence. FRET-efficiency values were binned for each selected time trace to generate histograms. The FRET-distribution histograms were fitted with the peak-fit function using Origin 8.5 software.

**Dwell time analysis**

Dwell times from high FRET states and low FRET states were obtained after analysis of FRET time traces with the HMM, and were integrated and normalized to produce distribution functions that were then fitted to simple exponential decay function, $\exp(-kt)$, or double exponential decay function, $A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t)$.

**DNA-cleavage assay**

The reaction buffer contained 8.5 nm negatively supercoiled pUC19 DNA and 50 nm DmTop2, added with either 1 mM ATP or AMPPNP in the presence or absence of 32 μM teniposide (VM26). Reaction mixtures were incubated at 30 °C for 30 min, and the reactions were terminated by adding SDS to a final concentration of 0.25%, EDTA to 10 mM, and proteinase K to 0.4 mg/ml. Incubation was continued at 50 °C for 1 h and then the samples were analyzed by electrophoresis in 1.2% agarose gels containing 0.5 μg/ml of ethidium bromide. As a positive control for DNA-cleavage, linearized pUC19 was generated by Scal digestion and loaded onto the gels alongside the reaction products.

**References**

1. Wang, J. C. (2002) Cellular roles of DNA topoisomerases: a molecular perspective. Nat. Rev. Mol. Cell Biol. 3, 430–440
2. Champoux, J. J. (2001) DNA topoisomerases: structure, function, and mechanism. Annu. Rev. Biochem. 70, 369–413
3. Nitiss, J. L. (2009) DNA topoisomerase II and its growing repertoire of biological functions. Nat. Rev. Cancer 9, 327–337
4. Pommier, Y., Sun, Y., Huang, S. N., and Nitiss, J. L. (2016) Roles of eukaryotic topoisomerases in transcription, replication and genomic stability. Nat. Rev. Mol. Cell Biol. 17, 703–721
5. Chen, S. H., Chan, N. L., and Hsieh, T. S. (2013) New mechanistic and functional insights into DNA topoisomerases. Annu. Rev. Biochem. 82, 139–170
6. Ashour, M. E., Atteya, R., and El-Khamisy, S. F. (2015) Topoisomerase-mediated chromosomal break repair: an emerging player in many games. Nat. Rev. Cancer 15, 137–151
7. Schoeffler, A. J., and Berger, J. M. (2008) DNA topoisomerases: harnessing and constraining energy to govern chromosome topology. Q Rev. Biophys. 41, 41–101
8. Nitiss, J. L. (2009) Targeting DNA topoisomerase II in cancer chemotherapy. Nat. Rev. Cancer 9, 338–350
9. Roca, J., Berger, J. M., Harrison, S. C., and Wang, J. C. (1996) DNA transport by a type II topoisomerase: direct evidence for a two-gate mechanism. Proc. Natl. Acad. Sci. U.S.A. 93, 4057–4062
10. Dong, K. C., and Berger, J. M. (2007) Structural basis for gate–DNA recognition and bending by type IIA topoisomerases. Nature 450, 1201–1205
11. Morris, S. K., and Lindsay, J. E. (1999) Yeast topoisomerase II is inhibited by etoposide after hydrolyzing the first ATP and before releasing the second ADP. J. Biol. Chem. 274, 30690–30696
12. Olland, S., and Wang, J. C. (1999) Catalysis of ATP hydrolysis by two NH₃-terminal fragments of yeast DNA topoisomerase II. J. Biol. Chem. 274, 21688–21694
13. Wu, C. C., Li, T. K., Farh, L., Lin, L. Y., Lin, T. S., Yu, Y. J., Yen, T. J., Chang, C. W., and Chan, N. L. (2011) Structural basis of type II topoisomerase inhibition by the anticancer drug etoposide. Science 333, 459–462
14. Roca, J., Ishida, R., Berger, J. M., Andoh, T., and Wang, J. C. (1994) Antitumor bisdioxopiperazines inhibit yeast DNA topoisomerase II by trapping the enzyme in the form of a closed protein clamp. Proc. Natl. Acad. Sci. U.S.A. 91, 1781–1785
15. Smiley, R. D., Collins, T. R., Hammers, G. G., and Hsieh, T. S. (2007) Single-molecule measurements of the opening and closing of the DNA gate by eukaryotic topoisomerase II. Proc. Natl. Acad. Sci. U.S.A. 104, 4840–4845
16. Collins, T. R., Hammers, G. G., and Hsieh, T. S. (2009) Analysis of the eukaryotic topoisomerase II DNA gate: a single-molecule FRET and structural perspective. Nucleic Acids Res. 37, 712–720
17. Lee, S., Jung, S. R., Heo, K., Byl, J. A., Deweese, J. E., Osheroff, N., and Hohng, S. (2012) DNA cleavage and opening reactions of human topoi-
somerase II are regulated via Mg\(^2\)+-mediated dynamic bending of gate-DNA. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 2925–2930
18. Hardin, A. H., Sarkar, S. K., Seol, Y., Liou, G. F., Osheroff, N., and Neuman, K. C. (2011) Direct measurement of DNA bending by type IIA topoisomerases: implications for non-equilibrium topology simplification. *Nucleic Acids Res.* **39**, 5729–5743
19. Deweese, J. E., and Osheroff, N. (2010) The use of divalent metal ions by type II topoisomerases. *Metallomics* **2**, 450–459
20. Sugino, A., Higgins, N. P., Brown, P. O., Peebles, C. L., and Cozzarelli, N. R. (1978) Energy coupling in DNA gyrase and the mechanism of action of novobiocin. *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4838–4842
21. Osheroff, N., Shelton, E. R., and Brutlag, D. L. (1983) DNA topoisomerase II from *Drosophila melanogaster*: relaxation of supercoiled DNA. *J. Biol. Chem.* **258**, 9536–9543
22. Roca, J., and Wang, J. C. (1992) The capture of a DNA double helix by an ATP-dependent protein clamp: a key step in DNA transport by type II DNA topoisomerases. *Cell* **71**, 833–840
23. Schmidt, B. H., Osheroff, N., and Berger, J. M. (2012) Structure of a topoisomerase II-DNA-nucleotide complex reveals a new control mechanism for ATPase activity. *Nat. Struct. Mol. Biol.* **19**, 1147–1154
24. Robinson, M. J., and Osheroff, N. (1991) Effects of antineoplastic drugs on the post-strand-passage DNA cleavage/religation equilibrium of topoisomerase II. *Biochemistry* **30**, 1807–1813
25. Schmidt, B. H., Burgin, A. B., Deweese, J. E., Osheroff, N., and Berger, J. M. (2010) A novel and unified two-metal mechanism for DNA cleavage by type II and IA topoisomerases. *Nature* **465**, 641–644
26. Baird, C. L., Harkins, T. T., Morris, S. K., and Lindsley, J. E. (2000) Pre-steady-state analysis of topoisomerase II trapped as the closed-clamp intermediate by ICRF-193. *J. Biol. Chem.* **275**, 2613–2618
27. Harkins, T. T., Lewis, T. J., and Lindsley, J. E. (1998) Pre-steady-state analysis of ATP hydrolysis by *Saccharomyces cerevisiae* DNA topoisomerase II: 2. kinetic mechanism for the sequential hydrolysis of two ATP. *Biochemistry* **37**, 7299–7312
28. Wendorff, T. J., Schmidt, B. H., Heslop, P., Austin, C. A., and Berger, J. M. (2012) The structure of DNA-bound human topoisomerase IIα: conformational mechanisms for coordinating inter-subunit interactions with DNA cleavage. *J. Mol. Biol.* **424**, 109–124
29. Harkins, T. T., and Lindsley, J. E. (1998) Pre-steady-state analysis of ATP hydrolysis by *Saccharomyces cerevisiae* DNA topoisomerase II: 1. a DNA-dependent burst in ATP hydrolysis. *Biochemistry* **37**, 7292–7298
30. Osheroff, N., Zechiedrich, E. L., and Gale, K. C. (1991) Catalytic function of DNA topoisomerase II. *Bioessays* **13**, 269–273
31. Jensen, L. H., Nitiss, K. C., Rose, A., Dong, J., Zhou, J., Hu, T., Osheroff, N., Jensen, P. B., Sehested, M., and Nitiss, J. L. (2000) A novel mechanism of cell killing by anti-topoisomerase II bisdioxopiperazines. *J. Biol. Chem.* **275**, 2137–2146
32. Wang, L., and Eastmond, D. A. (2002) Catalytic inhibitors of topoisomerase II are DNA-damaging agents: induction of chromosomal damage by merbarone and ICRF-187. *Environ. Mol. Mutagen.* **39**, 548–556
33. Huang, K. C., Gao, H., Yamasaki, E. F., Grabowski, D. R., Liu, S., Shen, L. L., Chan, K. K., Ganapathi, R., and Snapka, R. M. (2001) Topoisomerase II poisoning by ICRF-193. *J. Biol. Chem.* **276**, 44488–44494
34. Clasen, S., Olland, S., and Berger, J. M. (2003) Structure of the topoisomerase II ATPase region and its mechanism of inhibition by the chemotherapeutic agent ICRF-187. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 10629–10634
35. Morris, S. K., Baird, C. L., and Lindsley, J. E. (2000) Steady-state and rapid kinetic analysis of topoisomerase II trapped as the closed-clamp intermediate by ICRF-193. *J. Biol. Chem.* **275**, 2613–2618
36. Hu, T., Sage, H., and Hsieh, T. S. (2002) ATPase domain of eukaryotic DNA topoisomerase II: inhibition of ATPase activity by the anti-cancer drug bisdioxopiperazine and ATP/ADP-induced dimerization. *J. Biol. Chem.* **277**, 5944–5951
37. Bronson, J. F., Fei, J., Hofman, J. M., Gonzalez, R. L., Jr, and Wiggins, C. H. (2009) Learning rates and states from biophysical time series: a Bayesian approach to model selection and single-molecule FRET data. *Biophys. J.* **97**, 3196–3205