

Optical-Tweezers Study of Topoisomerase Inhibition**

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Topoisomerase is an especially interesting chemotherapy target, namely because of the topoisomerase-DNA complex, which undergoes mechanical motions essential to its function during the cleavage and religation of a single strand within a duplex DNA.^[1–6] Here we show the use of optical tweezers to study topoisomerase activity, evidenced by a large increase in the hysteresis of the force cycles resulting from the generation of ssDNA-like domains inside dsDNA, and subsequent strand religation indicated by recovery of the characteristic dsDNA force fingerprint. In contrast, the presence of the

topoisomerase inhibitor Lamellarin D^[7–9] results in a large increase in force hysteresis due to the initial nicking activity of Topo I and a subsequent absence of religation. These results highlight the potential of optical tweezers biosensors for the mechanistic study of DNA-modifying enzymes and for the screening of their inhibitors, and foresee profound implications in drug discovery and medical nanotechnology.

DNA-handling enzymes represent promising effectors of tumor proliferation as selective actuators against uncontrolled cellular growth. Topoisomerase (Topo) I from wheat germ^[1] is an eukaryotic type IB Topo^[2,3] that cleaves and rejoins one DNA strand through a covalent protein-DNA intermediate. The chemistry of this reaction involves nucleophilic attack of the phosphodiester backbone in duplex DNA by a tyrosine residue, which leads to a 3'-phosphotyrosyl linkage of the enzyme to one of the cleaved DNA strands.^[2] A free rotation model of action has been described for type IB Topo, in which individual catalytic cycles can alter the DNA linkage number by multiple integral turns, thereby relieving torsional stress.^[4] The distribution of cleavage sites in duplex DNA is non-random, but the principles governing cleavage site choice remain poorly understood. Topoisomerases are especially attractive targets for cancer therapy since their role in controlling DNA topology is crucial for correct cell division.^[3,5,6] Topoisomerase-targeted drugs are more selective for malignant cells, which are more susceptible to the DNA damage inflicted.^[6] Understanding the mechanism of action of the enzyme in the presence of its inhibitors is a requisite for the clinical development of therapeutic agents.

In this work, we used the widely known marine sponge alkaloid Lamellarin D (Lam-D, Figure 1)^[7–9] to study Topo I inhibition. Lam-D belongs to a group of anticancer drugs which act by poisoning and stabilizing DNA-Topo I phosphotyrosyl intermediates,^[8,10–12] a mechanism reported for numerous anticancer drugs.^[3,5,6,12–14] It has been proposed that interaction of the drug with the enzyme results in a ternary complex that inhibits post-cleavage DNA religation.^[13,15] In a previous structure-activity relationship study of Lam-D,^[16] we prepared a library of open lactone analogues of the natural product (open chain Lam-D, OCLam-D, Figure 1). GI₅₀ tested in a panel of three human tumor cell lines was significantly higher for OCLam-D when compared to Lam-D. In agreement with these data, AFM images of a plasmid DNA treated by Topo I in the presence of OCLam-D show all DNA molecules relaxed (Figure 2a,c,e), indicating poor enzyme inhibition. In contrast, Lam-D used at the same concentration is apparently able to block Topo I cleaving activity as indicated

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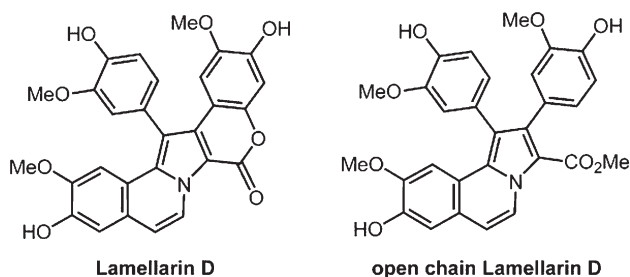


Figure 1. Chemical structures of Lamellarin D and of open chain Lamellarin D.

by the prevalence of supercoiled topoisomers (Figure 2b,d,f). However, AFM images do not permit to investigate the alternative inhibitory mechanism where Topo I would cleave but without releasing the newly generated DNA ends until completing the ligation step. To explore this scenario we have used an optical tweezers (OT) single molecule force spectroscopy (SMFS) approach.

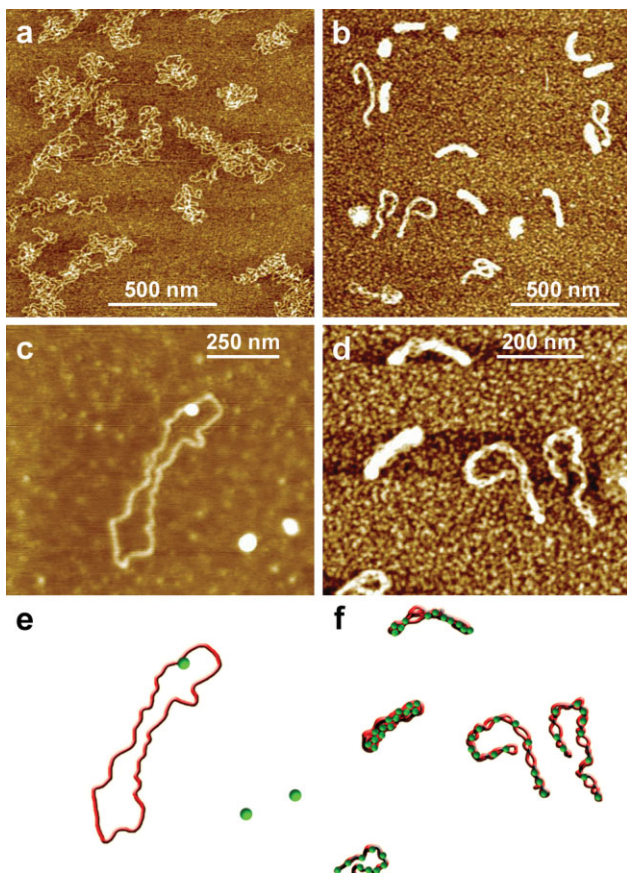


Figure 2. AFM imaging of Topo I-treated plasmid DNA. a,c) Supercoiled circular DNA treated by Topo I in the presence of OCLam-D, showing all DNA molecules relaxed as result of Topo I activity. b,d) Supercoiled circular DNA treated by Topo I in the presence of Lam-D. A similar number of molecules is shown in (a) and (b). The different apparent topoisomerase diameter and DNA width in (c) and (d) are the result of using AFM tips with different apex radii. e,f) Interpretive cartoon of the images from (c) and (d), respectively. Green dots represent topoisomerase molecules. Vertical color scale is 2 nm for all images.

Owing to their sub-piconewton force sensitivity, OT are a robust, powerful and highly sensitive biophysical tool which enables direct measurement of minute forces within biological engines such as Topo I. The lure of OT sensors resides in the fact that they provide biomolecular analysis of Topo I–DNA complexes at the smallest possible level, from which the enzymatic mechanism can be deduced as the sum of discrete phenomena. OT systems^[17–19] have been successfully employed as a SMFS biosensor to measure the elastic responses of immobilized DNA molecules^[20–22] and for the identification of molecular binding mechanisms.^[23]

When a linear dsDNA molecule is pulled from the 5' or 3' ends, its elastic response reaches a characteristic force plateau. This was first attributed to its change in structure from the B-form to the overstretched S-form.^[24] Based on data obtained at different ionic strengths, temperatures, and pH conditions,^[25] a model was proposed whereby the overstretching plateau was assigned to a force-induced melting process ending in short dsDNA domains holding large ssDNA strands together.^[25–27] In this overstretching transition plateau, further elongation results in an elastic response corresponding to a non-equilibrium process.^[28] Partial melting of the DNA molecule can occur at forces below 150 pN and is observed as a deviation of the relaxation path from the stretching path that depends on kinetic effects as well as on the number and location of nicks on the dsDNA.^[29] Therefore, the increase in hysteresis area can be used as a tight screening control of the nicking activity of Topo I.

Our OT setup employed a single linear dsDNA molecule immobilized between two streptavidin-coated microspheres through its 3' ends.^[21] We ran SMFS assays of this system alone and in the presence of Topo I (Figure 3a). Enzyme-induced changes in the DNA physical properties can be followed by plotting stretching-relaxing force cycles vs. DNA extension. Before Topo I addition, the individual dsDNA molecule held in the optical trap exhibits a stretch-retract profile that indicates the presence of strand breaks resulting from the DNA purification process (Figure 3b, black curve). The hysteresis curve of the 48.5-kbp λ DNA reaches a plateau at ca. 62 pN at 17 μ m, which marks the start of the melting process. The cleaving activity of Topo I was evidenced by a clear displacement of the stretching curve towards the ssDNA region (Figure 3b, red curve) as a result of the generation of single strand breaks that form ssDNA-like domains inside ds λ DNA. At the end of the experiment, the ssDNA-like domains were repaired via Topo I ligating activity, although nicks in the resulting dsDNA persisted, as indicated by the large hysteresis area remaining between the stretching and relax curves (Figure 3b, green curve).

Figure 3c shows another typical experiment where the λ DNA molecule held in the optical trap was cleaved by Topo I during the force cycle, as indicated by the sudden drop in the stretching curve (bold red, arrow). The formation of a higher level plateau (ca. 10 pN increase in force) and the total disappearance of hysteresis in stretching-relaxing cycles suggests that all nicks in the ds λ DNA were removed (green curve). The commonly reported plateau at ca. 62 pN likely reflects disruption of base pair stacking in dsDNA molecules having at least one nick, but the plateau just above 70 pN

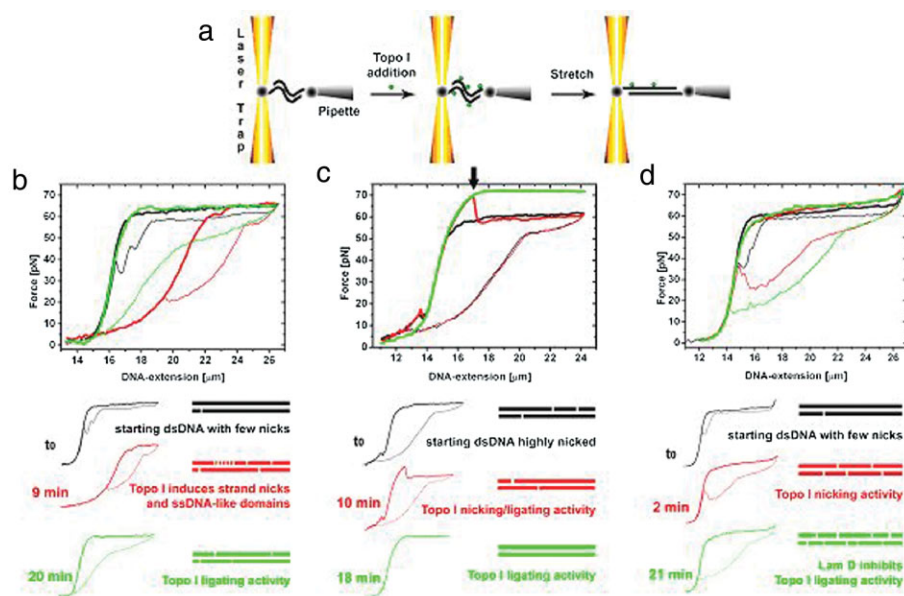


Figure 3. OT force assays of Topo I activity. a) Schematic of OT experiments, where each 3' biotinylated end of the dsDNA is attached to one streptavidin-coated bead. b) OT force-stretching experiment starting from a dsDNA molecule with few nicks, as indicated by the small hysteresis area (black curve). Stretching-relaxing cycles are indicated by half-bold half-thin plots. The cleaving activity of Topo I after 9 min of reaction generated an ssDNA-like domain, a heavily nicked dsDNA which exhibited the mechanical properties of ssDNA (red curve). After a further 11 min the ligating activity of Topo I removed the ssDNA-like domain (green curve). c) OT force-stretching experiment starting with highly nicked dsDNA, as indicated by the large hysteresis area (black curve). Topo I activity is evidenced by the presence of a higher level plateau that indicates total religation of the initial DNA nicks and a complete vanishing of the overstretching hysteresis (green curve, 18 min reaction time). The arrow indicates a drop in the stretching curve resulting from Topo I cleavage during the cycle. d) OT force assay of Topo I activity in the presence of Lam-D. Starting dsDNA exhibited a small hysteresis area (black curve) corresponding to a dsDNA molecule with only few nicks. The cleaving activity of Topo I induced a large increase in hysteresis in the presence of Lam-D after the first two minutes of reaction (red curve). Stabilization by Lam-D of this intermediate complex resulted in inhibition of the religating activity of Topo I (green curve, acquired at 21 min reaction).

corresponds to an intact dsDNA molecule without a single nick. This complete recovery of a dsDNA was usually achieved by Topo I when ssDNA domains had not been generated. Under our experimental conditions, in the presence of ssDNA domains (Figure 3b) the DNA seems to be too damaged to be completely repaired by Topo I. Occasionally, an experiment was ended when both dsDNA strands had been cleaved, an outcome consistent with reports on the formation of a Topo I dimer-like complex which cleaves dsDNA.^[30] In order to investigate the Topo I inhibition, experiments were performed as described above, except now in the presence of 10 μM Lam-D. The overall mechanic response of dsDNA molecules to this concentration of Lam-D showed a slightly tilted plateau deviation (Figure 3d), attributable to weak DNA intercalation of the drug, which agrees well with previously reported circular dichroism binding experiments.^[10] This small effect of Lam-D enabled accurate SMFS analysis of the complex in the presence of the enzyme.

OT results always and reproducibly exhibited a large increase in hysteresis of the force cycles due to the initial nicking activity of Topo I (Figure 3d, red curve). The presence of Lam-D prevents the religation step and blocks enzyme turnover, as evidenced by the absence of a higher force plateau

and by a large, non-vanishing hysteresis between the stretching and relaxing paths of the force cycle (Figure 3d, green curve). Taken together, the data presented here indicate that, upon Lam-D inhibition, Topo I keeps a non-covalent interaction with the 5' end of the cleaved DNA strand sufficiently strong to prevent supercoil relaxation in solution.

Single molecule approaches should prove paramount for further elucidation of the mechanisms of action of potential topoisomerase inhibitors such as Lam-D,^[8] namely, in enabling investigation of these molecules at pN sensitivity. We have shown that OT biosensor assays can be successfully applied to the study of the mechanism of Topo I inhibition by Lam-D at the single molecule level, representing a new approach to the study of the interaction between DNA binding enzymes and their inhibitors. We foresee that the use of OT-based screening to identify fundamental molecular mechanisms will have profound implications in biomolecular applications, drug discovery and medical nanotechnology.

Experimental Section

AFM imaging: Topo I activity assays were performed at 37 °C for 30 min in a 8- μl volume of reaction buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM (2S,3S)-1,4-bis-sulfanylbutane-2,3-diol) containing 1 μg of a 5-kbp plasmid DNA and 2.5 units of the enzyme (Sigma, Saint Louis, MI, USA). When required, 200 μM Lam-D or OCLam-D (synthesized^[9,16]) was included. For AFM imaging, the sample was diluted 10 times with reaction buffer. Mica surfaces (Provac AG, Balzers, Liechtenstein) were silanized in an exsiccator with (3-aminopropyl)triethoxysilane (Fluka, Steinheim, Germany).^[31] 5- μl diluted samples were deposited on the treated mica and let to adsorb for 15 min at room temperature, rinsed with deionized water (Millipore, Bedford, MA, USA) to remove weakly attached proteins, and finally dried under a gentle flow of nitrogen gas. Imaging was performed using non-contact aluminum-coated silicon nitride cantilevers with a spring constant of 40 Nm^{-1} and 300 kHz resonant frequency (Budget Sensors, Sofia, Bulgaria) on a Nanoscope IIIa AFM system equipped with a Multimode head and a type E piezoelectric scanner (Multimode, Veeco Instruments, Santa Barbara, CA, USA). The AFM was operated in tapping mode^[32] at a scan line frequency of 1–2 Hz. Raw AFM images have been processed only for background removal (flattening) using the microscope manufacturer's image-processing software. Image analysis by Fast Fourier Transforms

was performed with the WSxM 2.0 SPM software (Nanotec, Madrid, Spain).

Optical tweezers assays: Force measurements and manipulation of individual dsλDNA molecules in the presence of Topo I and Lam-D were performed with a compact single beam OT system equipped with a fluid cell for liquid handling. This setup was integrated into a commercial inverted optical microscope as previously described,^[21] and rebuilt for improved force resolution.^[33] A maximal and linear force detection range of 90 pN combined with a force sensitivity of less than 0.2 pN allowed measurements of elastic properties of single molecules. Streptavidin-coated polystyrene microspheres (Spherotech, Libertyville, IL, USA) with a diameter of 3.28 μm were used in a diluted suspension of 5 × 10⁻⁴ % w/v for all experiments. λDNA (Promega Corp., WI, USA) was chemically modified^[21] to ensure tethering to the beads. Beads, λDNA, and binding ligands were dissolved in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA reaction buffer. λDNA was used at a concentration of 15 μM, and Lam-D was used at a final concentration of 10 μM. 5 units of Topo I were used for each run. All experiments were performed at 20 °C.

Keywords:

atomic force microscopy · DNA · lamellarin D · optical tweezers · topoisomerase

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