Magnetic tweezers measurements of the nanomechanical properties of DNA in the presence of drugs

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ABSTRACT

Herein, we study the nanomechanical characteristics of single DNA molecules in the presence of DNA binders, including intercalating agents (ethidium bromide and doxorubicin), a minor groove binder (netropsin) and a typical alkylating damaging agent (cisplatin). We have used magnetic tweezers manipulation techniques, which allow us to measure the contour and persistence lengths together with the bending and torsional properties of DNA. For each drug, the specific variations of the nanomechanical properties induced in the DNA have been compared. We observed that the presence of drugs causes a specific variation in the DNA extension, a shift in the natural twist and a modification of bending dependence on the imposed twist. By introducing a naive model, we have justified an anomalous correlation of torsion data observed in the presence of intercalators. Finally, a data analysis criterion for discriminating between different molecular interactions among DNA and drugs has been suggested.

INTRODUCTION

Newly conceived anti-cancer drugs are usually scrutinized by considering their biological effects on mesoscopic samples in vivo or in vitro. Recently, new single molecule tools have been developed in order to test drug activity at the molecular level. Indeed, single molecule techniques have opened new ways for studying biologically and biomedically relevant problems because they are not limited to measuring only ensemble properties (1). Among single molecule techniques, the magnetic tweezers (MTs) technique is well suited to experiments on single DNA molecules (2,3). The MT technique allows us to manipulate small bodies or single macromolecules by connecting them to a micron-sized super-paramagnetic bead that, in turn, can be manipulated by means of macroscopic magnetic fields generated by permanent magnets or electromagnets (4). The DNA acts as a tether between the bead and a fixed position, which, in our case, is the inner surface of a capillary tube. Possible manipulations include stretching and torsion of the molecule. Simultaneous measurements of the bead position, applied force and imposed torsion give information about the nanomechanical properties of DNA, including bending and torsional properties (2,5).

Compared to other single molecule tools such as optical tweezers (OTs) (6–11), micropipettes (12) and atomic force microscopy (AFM) (3,13–15), the MTs method is much simpler to implement. Furthermore, the ability to measure torsional properties is a natural feature of MT. Incidentally, this ability has also recently been obtained with OT using unconventional nanoparticles and relative analysis (16,17).

The first pioneering MT studies addressed the topology of individual DNA molecules by measuring DNA extension as a function of the applied force and imposed torsion (18–20). In particular, it has been observed that torsion can produce plectonemes, which influence DNA extension (21). In some high-force or torsional stress conditions, nanomanipulation can also induce denaturation. Due to the chirality of DNA, this is obtained more easily with counterclockwise torsion than with clockwise torsion (2). Subsequently, MTs have been used in other configurations...
and in investigations of DNA in the presence of proteins, enzymes, ligands and drugs. For example, the activity of the gyrase enzyme has been studied by MT and has been demonstrated to exhibit three distinct modes of activity depending on the applied nanomechanical stress (22). Eukaryotic topoisomerase IB has been analyzed with MT by measuring the relaxation time of supercoiled DNA. In the presence of a topoisomerase, it has been shown that it is possible to evaluate the rotational friction of a DNA molecule around the single strand generated by the enzyme (23). On the basis of this work, the MT technique was then used to quantitatively measure the pharmaceutical efficacy of a well-known anti-cancer drug, camptothecin, which inhibits topoisomerase I activity (24).

Another enzyme, an RNA polymerase, has been studied by MT techniques; it was revealed that the initiation of the transcriptional process depends on the topology of the DNA (25,26). The effect of a specific anti-cancer drug, cisplatin, has been scrutinized by measuring force versus extension data obtained with MT techniques, and the obtained results have revealed the existence of two separate persistence lengths in kinked DNA in the presence of the drug (27).

Other similar reviews (28–30) have shown that MT techniques can provide an elegant description of single molecule aspects of DNA-ligand binding, which suggests that the use of MT could become a common technique for measuring a ligand’s effects on DNA.

We aim to use MT in order to compare the nanomechanical effects induced on DNA in the presence of four different ligands: ethidium bromide (ETBR), doxorubicin (DOXO), cisplatin (CIS) and netropsin (NETRO).

- ETBR is a DNA-intercalating molecule lacking antitumor activity that is used as a laboratory tool for DNA staining (31). It has been reported that ETBR modifies the DNA contour length (32–35) and induces unwinding (36,37).

- DOXO is a well-known DNA intercalating agent, and DNA is recognized as the primary target for its pharmacological action. Despite the central role of DNA binding in the antitumor activity of anthracyclines, available evidence indicates that the inhibition of a specific DNA function is responsible for their therapeutic effects. Indeed, the primary mechanism of cytotoxic and antitumor activity by DOXO is now ascribed to its interference with the function of topoisomerase II. DOXO functions as an enzyme poison by forming a DNA–drug–enzyme ternary complex, which thus stabilizes the cleavable complex in which DNA strands are broken and enzyme subunits are covalently linked to the DNA. Stabilization of the cleavable complex causes specific lethal DNA lesions (i.e. double-strand breaks) after collision with enzymes involved in DNA metabolism (38).

- CIS is an antitumor alkylating drug. CIS is a DNA-damaging agent that forms bifunctional adducts. The resultant cross-linked/damaged DNA causes cells to undergo apoptosis (39,40).

- NETRO is a minor groove binder that exhibits antiviral activity by inhibiting the replication of DNA and RNA viruses in mammalian cells (41). NETRO causes overtwisting of the DNA chain, as shown in (42–45).

In the following, we show the results of two different kinds of experiments. First, we measured the dependence of DNA extension on the applied force in the presence of these drugs. Then, we studied the effects of torsion. Both experiments were compared with control data obtained using bare DNA. The results were interpreted according to the available knowledge in the field, and we have experimental results that show new features that are reported here for the first time. Namely, in the case of intercalators, we observed an anomalous dependence in the extension versus twist data, which has been interpreted by introducing an extended naive mechanical model of DNA. Furthermore, we have extracted the dependence of the DNA bending characteristics as a function of twist, and we observed the unique behavior of different drugs. We suggest that these results may form the basis of a quantitative screening for anti-cancer drugs based on single molecule properties.

**MATERIALS AND METHODS**

**Drug characterization**

In this study, we used four different DNA binders: DOXO, ETBR (Sigma Aldrich), CIS (Bristol-Myers Squibb) and NETRO (Sigma Aldrich). All binders were diluted in 0.22 μm-filtered de-ionized pure water. For all of the MT measurements we used a fixed drug concentration (5 μM). This concentration is not enough to saturate all the binding sites, but it is sufficient to produce an observable effect on DNA nanomechanics characteristics.

**DNA preparation**

In the MT experiments, we used a 6964 bp DNA molecule suitably functionalized in order to provide a connection to a mobile support (the magnetic bead) at one end and to an immobile support (the capillary tube) at the other end. It is important to note that the DNA is attached via multiple bonds to both of the supports, which means that the molecule is torsionally constrained and that the magnetic rotation of the bead induces a real torque on the DNA because it cannot swivel around the bonds (2).

To prepare this construct, the DNA tails were functionalized at one end with biotin, for linking to an avidin-coated magnetic microparticle, and at the other end with digoxigenin, for linking to an anti-digoxigenin-coated capillary. The central portion of the DNA consists of a 5780 bp pCMV6-neo plasmid (Origene), while the ends are 537 and 647 bp stretches of the pBR322 plasmid (Stratagene) obtained using a PCR reaction with biotin or digoxigenin-labeled nucleotides, respectively. PCR reactions were performed using two oligonucleotides annealed to the pBR322 plasmid and carrying the restriction enzyme sites NotI (5'-CATATGCGGGCCGC ATGCAGCAGTCCTCGCATGACACTTCCCTTG...
PCR was performed in the presence of biotin-16-dUTP or digoxigenin-11-dUTP-labeled nucleotides (Roche). The PCR conditions were as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 1 min, 58°C for 1 min and 72°C for 1 min, and then 72°C for 5 min. The two products of the PCR amplification were restricted using the NotI and SacII enzymes and gel purified on an 0.8% agarose gel (Qiagen). The three DNA molecules were then ligated using a ligase reaction (T4 DNA ligase, Stratagene), and we thereby obtained the functionalized DNA solution with a total of 10^7 molecules.

After a 5-min incubation, the solution was diluted with 100 μl of 10 mM PBS at pH 8 supplemented with 0.1% Tween-20. The end result was dispersed in a volume of about 10 µl. To this solution, we added 10 µl of functionalized DNA solution with a total of 10^7 molecules. After a 5-min incubation, the solution was diluted with 500 µl of 10 mM PBS at pH 8 supplemented with 0.1% Tween-20 and 3 mM NaN3 (referred to hereafter as PBS-Tween-20).

**Cell preparation**

The modified DNA and magnetic beads were studied in a flow cell made of a properly functionalized capillary. For each measurement session, 250 μl of the prepared suspension (modified DNA and magnetic beads) was injected, in the absence of a magnetic field, into a glass capillary tube (1 × 1 mm² section, 5-cm long, VitroCom, Mountain Lakes, NJ) that had been functionalized as follows. First, a solution of 100 mg/ml polystyrene (average Mw ≈ 230000, Aldrich) in toluene (purum, Riedel-de Haén) was injected into the capillary tubes. Then, the capillaries were drained and blown with compressed air. In this way, the internal walls were uniformly coated with polystyrene (46). Next, 5 μg of sheep polyclonal anti-digoxigenin antibody (Roche) was incubated in the capillary tubes for 2 h at 37°C in 100 μl with 10 mM PBS. Unbound anti-digoxigenin was eliminated by rinsing the tubes with PBS-Tween-20. The treated surface was passivated for 2 h at 37°C with a solution consisting of 10 mM PBS at pH 8 supplemented with 0.1% Tween-20, 1 mg/ml fish sperm DNA (Roche) and 3 mM NaN3 (47). Finally, the capillary tubes were rinsed with PBS-Tween-20 and incubated for ~1h to allow the DNA to bind to the lower capillary surface. For storage, the tubes were dried with compressed air and kept at −20°C.

**MTs technique**

The MTs setup was assembled according to other similar apparatus reported in the literature (2,48). The capillary, linked to a buffer-flow system, was placed over the objective (Nikon 100× 1.25NA oil immersion with a 15-cm-focal-length tube lens, which leads to an actual magnification of 75x) of an inverted microscope equipped with a piezoelectric focusing system (PIFoc, Physik Instrumente). Images were acquired at a rate of 40 frames per second with a CCD camera (Marlin, Allied Vision Technologies) and were fed into a PC. From these data, we extracted the time dependence of the x, y and z positions of the magnetic bead. The magnetic field used to pull and twist the beads was generated by two cubic, 5 mm neodymium magnets with a 2-mm gap between them. A permalloy ring was placed outside the magnets to convey the magnetic field between the outer faces of the magnets. The configuration of the magnets has been previously discussed (49,50), and here we used a setup similar to that shown in Figure 2 of (49), which corresponds to the model presented in Figure 1b of (50). The magnets can be lowered (raised) to increase (decrease) the stretching force acting on the DNA. Due to the permanent magnetic moment of the bead, a rotation of the magnets around their axis induces a rotation of the bead, which induces torsion on the DNA molecules.

**Data analysis**

Using our MT setup, we measured three quantities: DNA extension, applied force and number of imposed turns.

DNA extension was calculated by considering the diffraction images generated at different heights of the bead (51,52). After obtaining a series of calibrating images at known positions, the actual position is obtained by comparing the acquired images to the calibrated images through a fitting algorithm.

To evaluate the force exerted on the beads by the magnetic field, we measured the mean square displacement σ^2 of the horizontal position along the x-axis (2,53) at each magnet height,

σ^2 = ⟨(x − ⟨x⟩)^2⟩ (1)

The variance in the bead excursion was evaluated considering the camera integration time and accordingly corrected (29). Then, we extracted the force F using the equipartition theorem (54)

F = k_B T L_e σ^2 (2)

where k_B is the Boltzmann constant, T the temperature and L_e the DNA extension. Once the system had been calibrated, we were able to apply a known force ranging from a few fN to several pN.

Representative measurements of force F versus extension L_e obtained by the MTs technique on bare DNA are shown in Figure 1c. We concentrated our attention on the behavior of DNA at relatively low applied forces F (F < 10 pN), which is the so-called ‘entropic’ regime (2). In such a regime, the external force generated by the magnets is...
counter-balanced by the decrease in entropy caused by a reduction in the number of available configurations of the DNA molecule. As shown in Figure 1c, in this regime, \( L_e \) is an increasing function of \( F \), and the specific shape of the curve can be fitted by the worm-like chain model (WLC) (2). In this model, DNA is treated as a homogeneous, isotropic polymer with a specific value of the bending stiffness \( B \). Accordingly, \( F \) versus \( L_e \) data are described by the following approximate interpolation formula, which considers the contour length \( L_0 \) and the persistence length \( L_p \) as free parameters

\[
\frac{F L_p}{k_B T} = \frac{1}{4} \left( 1 - \frac{L_e}{L_0} \right)^2 - \frac{1}{4} + \frac{L_e}{L_0}
\]

Thus, \( L_p = B(k_B T) \) influences the vertical shift of the force versus extension curve, while \( L_0 \) is related to the maximum asymptotic length attained by DNA at high force (29). In Figure 1a, we show a sketch describing what happens to DNA in two configurations consisting of high and low mechanical stress. As shown in Figure 1a, when the magnet is lowered, the force becomes stronger and the DNA is highly stretched by the magnetic force acting on the bead. As shown in Figure 1c, by fitting the data with Equation 3, the WLC accurately describes the data and gives values for the free parameters of \( L_0 = 1.8 \pm 0.1 \mu m \) and \( L_p = 45 \pm 3 \text{nm} \). The value of the contour length appears to be compatible with the number of DNA base pairs when considering a sedimentation height of a few hundreds of nanometers.

By rotating the external magnets at a fixed height, the MT technique creates the possibility of applying a torque to torsionally constrained DNA. We have exploited this capability by measuring the extension \( L_e \) of a DNA molecule as a function of the number of imposed turns \( n_t \). The obtained \( L_e \) versus \( n_t \) data are shown in Figure 1d for the representative case of bare DNA and for two different values of the applied pulling force. At low force values (yellow and red dots in Figure 1d), the \( L_e \) versus \( n_t \) data are symmetric, while at high force (see green and black dots in Figure 1d), the clockwise and anticlockwise rotations induce different behaviors due to the intrinsically chiral nature of DNA. Specifically, at low force values, we can distinguish between two different regions in the measured data: a central ‘low turn’ region (yellow dots in Figure 1d) where \( L_e \) is basically constant and two lateral ‘high turn’ regions (red dots in Figure 1d) where \( L_e \) is linearly dependent (increasing or decreasing) on \( n_t \).

This behavior is qualitatively described by the sketch of Figure 1b. Starting from a relaxed configuration, the DNA molecule becomes more and more tangled as
the magnets rotate, and eventually plectonemes are formed. The formation of plectonemes induces a progressive linear decrease in the DNA extension, which is analogous to the decrease in extension of a twisted and tangled telephone cord.

Indeed, the topology of a torsionally constrained segment of DNA is described by two quantities: the twist number \( T_w = N_{bp}/h \) (where \( N_{bp} \) is the base pair number and \( h \) the helical pitch), which corresponds to the number of helical turns in the double helix, and the writhe number \( W_r \), which is defined as the number of times the axis of the double helix crosses itself (2). In the absence of external torque, the natural twist of DNA is \( T_{W0} \), which is obtained by considering the natural value of \( h \) (\( h_0 = 10.4 \) bp per turn), with \( W_{T0} = 0 \). Since the torsion is initially absorbed by the elastic twist deformations, the application of a torque first implies an increase in \( T_w \), while \( W_r \) remains constant. This corresponds to the ‘low turn’ central and symmetric region, where the DNA extension is approximately constant and does not depend on \( n_t \) (yellow dots in Figure 1d). As the torque is continuously increased, the DNA undergoes a buckling transition at a specific number of imposed turns \( n_b \). At this point, loops or plectonemes, are formed (see Figure 1d, where \( n_b \) is indicated by the vertical arrows). Indeed, beyond the \( n_b \) number, it is energetically convenient to release energy from the elastic twisting deformations by bending the double helix, forming plectonemes, and increasing \( W_r \) instead of increasing the \( T_w \) number. Above \( n_b \), the DNA extension decreases linearly with \( n_b \), with a slope \( \Delta z \) (see the blue fitting line in Figure 1d). Both the buckling number \( n_b \) and the slope \( \Delta z \) in the plectonemic region depend on the values of the applied force. Finally, by considering negative imposed turns and high force values (green dots in Figure 1d), the DNA extension is no more dependent on the rotation, because the torsion energy is mainly absorbed by denaturing (instead of writhing) the DNA and inducing the formation of denaturation ‘bubbles’ (2).

Furthermore, in the frame of the quoted naive model (29), the double-stranded DNA is simply represented as an elastic cylinder with a torsional constant \( C \) and a bending constant \( B \). By minimizing the elastic energy and considering only positive values of rotations \( n_t > 0 \), the model (2,48) predicts the following expressions for \( n_b \) and \( \Delta z \):

\[
\begin{align*}
n_b &= \frac{L_0}{\pi C} \sqrt{BF} \\
\Delta z &= 2\pi \sqrt{\frac{B}{2F}}
\end{align*}
\]

According to this simplified theory, we should expect a sharp transition between the twisting region (constant DNA extension) and the plectonemic region (linearly decreasing DNA extension). In contrast, in our experiment, we observed a smooth transition between the two trends. This previously observed discrepancy is normally attributed to thermal noise or to oversimplifications in the model (2). Due to this smoothing of the curve at the transition, \( n_b \) has been extracted as the intersection value between the linear fitting of the plectonemic region and a horizontal line intercepting the maximum extension value.

We point out that there are more rigorous mechanical models of the buckling and post-buckling behavior of chains under torsion (55–58). However, we think that the naive model is still valuable given our goal of obtaining a qualitative discrimination between different molecular interaction.

**RESULTS AND DISCUSSION**

**Extension versus force data**

To describe the mechanical modifications induced by the drugs’ interaction with the DNA, we first studied the DNA extension. The results are shown in Figure 2, where we have plotted the \( F \) versus \( L_e \) data obtained with the above-described MTs technique. The measurement protocol was as follows: after binding the DNA to the bead and the capillary, we first applied an increasing force and measured the resulting extension in the absence of any drugs. This procedure gives a control measurement without drugs, which was compared to the data obtained with drugs. Next, we decreased the force by increasing the height of the magnets. Then, in the absence of applied force, we injected the drug under investigation into the capillary and measured the corresponding new DNA extension as a function of the applied force. As is apparent in the graph (Figure 2) and by considering the asymptotic length attained by the DNA at high forces, we observed that, in some cases, the extension is increased (DOXO and ETBR, length increment of \( \sim 30\% \)), while in other cases, the extension is reduced (CIS, length decrement of \( \sim 15\% \)). With NETRO, no relevant variations were observed. These results are easily understood by considering the molecular effects of the added drugs, and they confirm other recently published data in the literature obtained with either similar or different techniques (27,59–61). Indeed, DOXO and ETBR, two intercalators that have
comparable chemical DNA-binding behavior, induce an increase of $\Delta L = 0.34\text{nm}$ for each bond (13). In contrast, the binding of CIS introduces a kink in the DNA molecules, which does not affect the contour length but reduces the maximum possible extension of the DNA (62). As a result of its mechanism of binding to DNA (minor groove binder), NETRO has no relevant effect on the extension of the nucleic acid. The results, obtained by fitting the data presented in Figure 2 with Equation 3, are summarized in Table 1, where we have shown the measured DNA total contour length increment $\Delta L_0 = L_0(\text{DNA+drug}) - L_0(\text{bareDNA})$, the length increment due to a single bound drug molecule $\Delta L$ (as derived from the literature (13)), and the derived total number of binding events $N_F = \Delta L_0 / \Delta L$.

**Table 1. $F$ versus $L_e$ results**

| Drug | $\Delta L_0$ (nm) | $\Delta L$ (nm) | $N_F$ |
|------|------------------|----------------|-------|
| ETBR | 780              | $\approx 0.34$ | $\approx 2300$ |
| DOXO | 550              | $\approx 0.34$ | $\approx 1600$ |

$\Delta L_e$ corresponds to the total contour length increment induced by the ligands on the DNA, $\Delta L$ corresponds to the length increment induced by a single bound molecule as derived from the literature [13], and $N_F$ is the estimated number of bound molecules.

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**Extension versus imposed turns**

To further explore the nanomechanical effects induced by drugs, we also imposed a torque on the DNA and studied the extension versus number of turns in several experimental situations. The results are shown in Figure 3, where we have illustrated the MT results obtained at low force ($F = 0.16\text{pN}$) for bare DNA and in the presence of the tested drugs. Compared to the bare DNA, the addition of drugs caused a lateral positive or negative shift (indicated in Figure 3 by the arrows labeled $n_{t_0}$) in the $L_e$ versus $n_t$ data. The intercalators (DOXO and ETBR) and the alkylation agent (CIS) induce a negative shift ($T_w < T_w^0$), while the minor groove binder (NETRO) induces a positive shift ($T_w > T_w^0$). In this case, the measurement protocol was slightly modified: after the control check, we injected the drug while keeping the applied force constant (i.e. without any variation in the height of the magnets). Without this procedure, it would have been impossible to measure the shift in the twisting number $T_w$ with respect to the control value.

In the bare DNA, the number of turns $n_t = 0$ corresponds to the natural value of twist $T_w = T_w^0$, and the extension versus turn data are symmetric with respect to $n_t = 0$. The shift observed in the presence of drugs is due to a variation in the helical pitch. Indeed, the DOXO and ETBR molecules bind between two nucleobase pairs, and they relax the natural twist of a fixed angle by $-23^\circ$ and $-140^\circ$ for ETBR and CIS, respectively.

**Figure 3.** DNA extension $L_e$ measured as a function of the imposed turns $n_t$ for bare DNA and in the presence of various drugs (see legend). Data were taken at a fixed applied force $F = 0.16\text{pN}$ using the protocol described in the text. $n_{t_0}$ represents the shift in the twisting number $T_w$ induced by the drugs. The continuous lines are a guide to the eye.
−27°, respectively, per molecule (13,36). In contrast, and as expected (42), NETRO overtwists the double helix by 8°. The case of CIS is different because there are three different available sites for DNA binding. Each site corresponds to a different relaxation angle and has a different probability of occupation (63,64). There is a 65% probability of CIS binding to a G–G sequence and inducing a twist of 13°, a 25% probability of binding to an A–G sequence and inducing a twist of 13°, and a 10% probability of binding to a G–X–G sequence and inducing a twist of 25°. By a simple evaluation of the statistical distribution of the number of bound CIS molecules for any possible combination of binding sites, it is possible to predict the resulting twisting number. Considering a random sequence of 5780 bp, the twisting number is expected to be between 54 and 64 turns, which is in reasonable agreement with the observed value ($n_0 = 52$). Table 2 shows, for each drug, the measured total twist $n_0$, the twist angle $\Delta n$ due to a single binding event as derived from the literature (36, 42, 63–66), and the derived total number $N_T = n_0/\Delta n$ of bound molecules.

**Effect of twist on contour length**

The above-described experiments (Figure 3) were performed at a low value of applied force, where the DNA torsional behavior is symmetric. We also systematically studied the dependence of $L_e$ versus $n_t$ on the applied force. In Figure 4, we show two representative examples obtained in the presence of ETBR (left) and CIS (right). Considering the analogous data obtained for DNA alone (Figure 1d), we observe that the ETBR measurements (and DOXO, data not shown) present unexpected behavior. Indeed, in the twisting central region (before $n_b$, shown by the ellipses in Figure 4), an anomalous negative slope is present in the data for $L_e$ versus $n_t$. If we extend the naive model presented in the ‘Materials and Methods’ section, this observation can be explained in a very simplified way by considering the double helix as a string (of backbone length $L_{Helix}$) wrapped around a cylindrical inner core (of radius $R$) (Figure 5). The linear extension $L_o$ of the string depends, for a fixed

|       | $N_{to}$ | $\Delta n$ | $N_T$  |
|-------|----------|------------|--------|
| ETBR  | −85      | −27°       | ±1130  |
| CIS   | −52      | −13° & 25° | ±500   |
| DOXO  | −41      | −11°       | ±1340  |
| NETRO | 12       | 8°         | ±540   |

$n_{to}$ corresponds to the total twist induced by the ligands on the DNA, $\Delta n$ corresponds to the twist angle induced by a single bound molecule as derived from the literature (36, 42, 63–66), and $N_T$ is the estimated number of bound molecules.

![Figure 4](image-url) DNA extension $L_e$ measured as a function of the imposed turns $n_t$ for ETBR (a) and CIS (b) Data taken various applied force $F$, (squares: $F = 0.16\text{pN}$; stars: $F = 0.26\text{pN}$; circles: $F = 0.62\text{pN}$; diamonds: $F = 2.96\text{pN}$). The vertical arrows and the blue lines are the same as for Figure 1d. The ellipses in (a) emphasize an anomalous slope in the data; see text for details. The horizontal double arrows indicate the values of $n_b$ and $\Delta Tw$ for the representative case of $F = 0.16\text{pN}$. 

Table 2. $L_e$ versus $n_t$ results
radius, on the twist number as expressed by the following formula:

\[ L_0 = \sqrt{L_{\text{Helix}}^2 - (2\pi RTw)^2} \]  \hspace{1cm} (6)

where the relationship between \( T_w \) and \( n_t \) is the following:

\[
\begin{align*}
&T_w - T_{w0} \equiv \Delta T_w = n_t, \quad \text{if} \quad n_t < n_{00} + n_b \\
&T_w - T_{w0} \equiv \Delta T_w = n_{00} + n_b, \quad \text{if} \quad n_t \geq n_{00} + n_b
\end{align*}
\]  \hspace{1cm} (7)

According to this extension of the naive model, in the absence of ligands, the cylinder is empty and is taken to be compressible. Then, at a low number of imposed turns, the torsion simply induces a shrinkage of the DNA core while keeping \( L_c \), and as consequence \( L_c \) constant (see Figure 5b). Conversely, in the presence of intercalators, the radius \( R \) can be considered constant due to the steric hindrance of the drugs. Such ligands induce a reduction of the inner core’s compressibility; thus, an increase in the turn number requires a decrease in the DNA extension (see Figure 5c). The anomalous slope depends on the binding characteristics of the drug under investigation: ligands acting as intercalators increase the incompressibility of the DNA inner core, while minor groove binders and alkylating agents do not have this capability. The slope of the extension versus turn data in the central region is compatible, in our simplified model, with an inner core diameter \( 2R \approx 0.8 \text{ nm} \) (see formula (6)), which is similar to the thickness of a nucleobase pair.

**Analysis of the plectonemic region**

Considering now the plectonemic region (the linearly decreasing region of data in Figure 4), by exploiting the fitting method described in the Materials and Methods section, for each drug and for each applied force it is possible to obtain an evaluation of the buckling number \( n_b \) and the slope \( \Delta z \). The resulting data are shown in Figure 6 for the two representative cases of ETBR and CIS as a function of the force \( F \). The dependence of \( n_b \) as a function of \( F \) (Figure 6a) appears to be reasonably well described by a power law \( (n_b \propto F^\alpha) \) with exponent \( \alpha \). This power law behavior is also confirmed for all of the other investigated drugs (data not shown), and in Table 3, we have reported the corresponding values of \( \alpha \) obtained by the fitting procedure.

Analogously, we have plotted \( \Delta z \) as a function of the applied force \( F \) (Figure 6), and the data appear basically constant. All of the drugs under investigation (data not shown) show a similar constant behavior or a slightly negative power law \( (\Delta z \propto F^{\beta}) \) with a value of the exponent \( \beta \) close to zero (see Table 3).

It should be noted that the naive mechanical model predicts a specific power law dependence of \( n_b \) and \( \Delta z \) as a function of the applied force \( (n_b \propto F^{1/2}; \Delta z \propto F^{1/2}) \), see Equation. (4)&(5) This prediction is not confirmed by the data, as previously reported in the literature (2,55–58). This discrepancy is not really surprising, considering the reasonable possibility that \( B \) increases with the imposed overtwist. More specifically, the \( n_b \) and \( \Delta z \) parameters, according to the simplified model, are obtained by minimizing the energy resulting from three combined terms: the energy required to pull the bead, the energy to bend the DNA, and the energy to twist the double helix. The buckling instability occurs when the third factor is larger than the sum of the other two. At larger forces, the value of the first two factors increases and induces an increase in \( n_b \), as observed in all of the experiments. On the other hand, the two contributions that determine \( \Delta z \) are opposite, and the resulting behavior is neither trivial nor predictable by simple...
heuristic considerations. In any case, after the buckling instability is reached, any further turns imposed on the DNA do not increase its twist number ($T_w$) but instead increase the number of formed plectonemes ($W_r$ number) while keeping $B$ and $C$ fixed. We have assumed local validity of the naive model in the plectonemic region (for $n_t > n_b$) where the twist of the DNA is constant. Accordingly, we have considered Equation (5), and by simply solving it for $B$, we obtained:

$$B = F \Delta z^2/(2\pi^2).$$

Then, from the data shown in Figure 6, we have derived a qualitative estimate of $B$ for all of the applied forces. The obtained $B$ values are shown in Figure 7 as functions of the absolute twist difference with respect to the bare DNA, $\Delta T_w = T_w - T_{w_0}$. As is apparent in Figure 7, the DNA bending characteristics depend on the imposed overtwist, and the different drugs result in different behaviors. The two intercalators (ETBR and DOXO) show a mild dependence of $B$ on $\Delta T_w$ due to the initial DNA elongation induced by the drug. The minor groove binder and alkylating agent studied (NETRO and CIS) do not modify the stronger dependence of $B$ on $\Delta T_w$ observed with bare DNA, but their data are shifted to the positive (NETRO) and negative (CIS) regions of the plot, i.e. for positive or negative $\Delta T_w$. Indeed, the relevant result shown in Figure 7 is the fact that this analysis allows us to easily and qualitatively distinguish among the different families of drugs. While all of the previous data analyses are not truly able to discriminate between different interaction mechanisms of drugs, we suggest that the consideration of $B$ versus $\Delta T_w$ allows one to make a distinction between various binding characteristics.
CONCLUSIONS

In this article, we have measured some nanomechanical properties of DNA in the presence of various binding molecules by means of MTs techniques. We have observed that the presence of drugs induces a variation in the DNA length and a shift in the extension versus turn data. In the case of intercalators, we have also observed an anomalous dependence of DNA extension on the number of imposed turns that is not due to plectonemic formation. This result has been qualitatively justified by considering the molecular interaction mechanisms involved in the binding phenomenon and by assuming a simple excluded volume model. Finally, we have observed that the DNA bending characteristics depend on the imposed turn number. Intercalators are able to modify this dependence, while minor groove binders and alkylating agents cannot. We suggest that this dependence indicates a general method for classifying different DNA-binding molecules. This discrimination can be considered as a particularly relevant result because this methodology could allow for a rapid and detailed screening of pharmacologically active drugs. DNA is recognized as the primary target of many established antitumor agents. The efficacy of DNA-interacting agents is related to their direct effects on DNA structure or to the consequences of their binding on DNA functions. It is evident that not all DNA-interacting agents are useful as therapeutic drugs. Although the efficacy of a DNA-interacting agent is not simply related to its ability to bind DNA, its pharmacodynamic properties clearly reflect its mode of DNA-binding and its influence on DNA structure (e.g. DNA damage). Efforts focusing on rational approaches to predict optimal DNA interactions may have useful implications.

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