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Binding mechanism of anti-cancer chemotherapeutic drug mitoxantrone to DNA characterized by magnetic tweezers

Dennis Kreft¹, Ying Wang¹, Michael Rattay², Katja Toensing¹ and Dario Anselmetti^{1*}

Abstract

Background: Chemotherapeutic agents (anti-cancer drugs) are small cytostatic or cytotoxic molecules that often bind to double-stranded DNA (dsDNA) resulting in modifications of their structural and nanomechanical properties and thus interfering with the cell proliferation process.

Methods: We investigated the anthraquinone compound mitoxantrone that is used for treating certain cancer types like leukemia and lymphoma with magnetic tweezers as a single molecule nanosensor. In order to study the association of mitoxantrone with dsDNA, we conducted force-extension and mechanical overwinding experiments with a sensitivity of 10^{-14} N.

Results: Using this method, we were able to estimate an equilibrium constant of association $K_a \approx 1 \times 10^5 \text{ M}^{-1}$ as well as a binding site size of $n \approx 2.5$ base pairs for mitoxantrone. An unwinding angle of mitoxantrone-intercalation of $\vartheta \approx 16^\circ$ was determined.

Conclusion: Moreover, we observed a complex concentration-dependent bimodal binding behavior, where mitoxantrone associates to dsDNA as an intercalator and groove binder simultaneously at low concentrations and as a mere intercalator at high concentrations.

Keywords: Mitoxantrone, DNA, Magnetic tweezers, Intercalator, Groove binder

Background

Regarding the high morbidity and mortality rate of cancer diseases in the recent decades, the development of cytostatic and cytotoxic chemotherapeutics is highly promoted. Several types of such anti-tumor agents, e.g. anthracycline, bind to DNA polymers in tumor/cancer cells and consequently result in an inhibition of cell growth (cytostatic/antiproliferative activity) or even necrosis (cytotoxic activity). Their heal efficacy depends strongly on the binding mode and the nanomechanism of the DNA-drug interaction. Therefore, a deep and thorough understanding of these biophysical characteristics

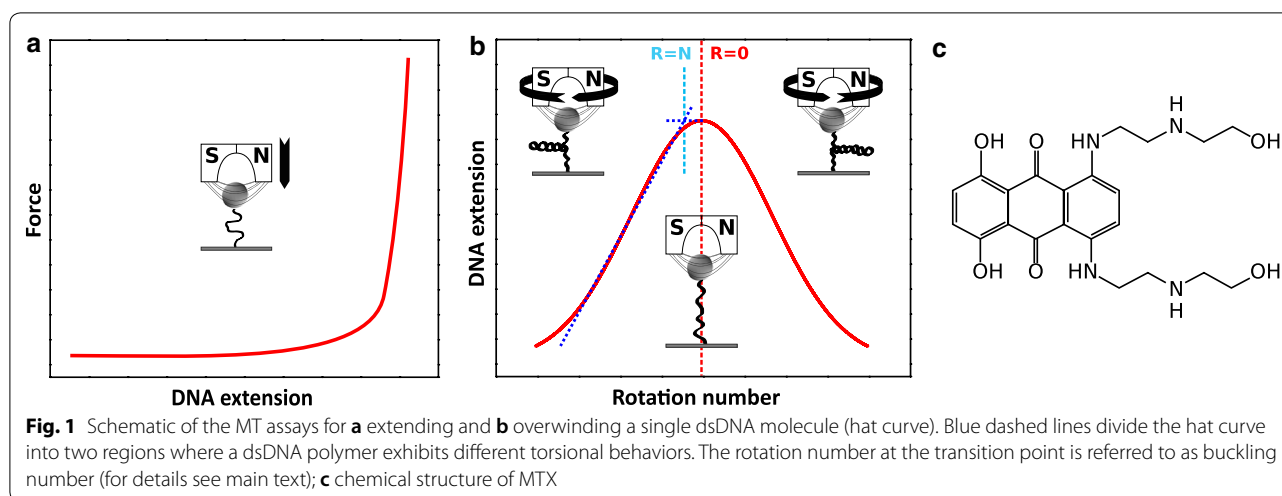
of chemotherapeutics in the perspective of molecular recognition contributes significantly to the medical regulation and optimization of pharmaceuticals.

Here, we focused on an anthraquinone derivative mitoxantrone (MTX, 1,4-dihydroxy-5,8-bis[2-(2-hydroxyethylamino)ethylamino]anthracene-9,10-dione, chemical structure see Fig. 1c [1]). The topoisomerase II-inhibitor MTX was first synthesized in the late 1970s by Zee-Cheng and Cheng and Murdock et al. independently [2–4]. As a promising chemotherapeutics, MTX is broadly used in the treatment of different cancers such as metastatic breast cancer and acute lymphoblastic leukemia as well as of multiple sclerosis [5–9]. Compared to other members of the anthracycline family, MTX has a comparable cytostatic activity but lower cardiotoxicity [10–13]. Besides the medical applications, the binding of MTX to DNA and its corresponding influence on the nanomechanical and structural properties of DNA

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are still not fully understood. MTX was well-known to bind to DNA as a classical intercalator. However, several publications pointed out an additional groove-binding of MTX [14–20]. The quantifications of the binding mechanism of MTX are also not very consistent. Kapuscinski et al. reported a binding affinity of the MTX-DNA interaction of $K_a \sim 10^5 \text{ M}^{-1}$ [21], whereas other research groups estimated the value one order of magnitude higher [15, 18, 22–25]. Furthermore, DNA-untwisting due to MTX-intercalation was hardly studied. In this work, we performed single molecule nanosensor magnetic tweezers (MT) experiments to investigate the association of MTX with dsDNA. By means of extending and overwinding experiments within a force range of 0.005–10 pN, we analyzed the effects of MTX-binding on the nanomechanical and structural properties of dsDNA e.g. elongation, softening and unwinding. As a result, we categorized the MTX-dsDNA association as a complex concentration-dependent bimodal binding.

Methods

For the performance of MT-experiments (Fig. 1a/b), we used a commercial MT system (PicoTwist, Lyon, France) with a self-made microfluidic flow cell. The experimental setup and flow cell assembly were previously described in detail [26–31]. In brief, the surface of the flow cell was covalently coated with sigmacote (Sigma-Aldrich, Hamburg, Germany) for a homogeneous hydrophobic surface and subsequently functionalized with anti-digoxigenin (200 µg/ml, Roche, Penzberg, Germany). For MT experiments, we prepared λ-dsDNA fragments which were functionalized at one end with several biotins (Biotin-14-dCTP, Metabion, Steinkirchen, Germany) and with several digoxigenins (Dig-11-dUTP, Roche, Penzberg, Germany) at the other end according to a published

protocol [29, 32, 33]. The 11.8 kbp fragments, corresponding to a contour length of about 4 µm, were separated by gel electrophoresis. Via the specific bonds, single dsDNA molecules were attached between the anti-dig functionalized surface and streptavidin coated superparamagnetic beads with a diameter of 1 µm (Dynabeads MyOne, Thermo Fisher Scientific, Waltham, USA). As a reference and control for each investigated DNA molecule, we verified its contour- and persistence length by means of stretching experiments and approximation of the force-extension curves to the worm-like-chain (WLC) polymer elasticity model [34, 35]:

$$\frac{FP}{k_B T} = \frac{1}{4} \left(\left(1 - \frac{d}{L(c)} \right)^{-2} - 1 \right) + \frac{d}{L(c)} \quad (1)$$

Here, F , P , $L(c)$, $k_B T$ and d represent the applied force, dsDNA persistence length, dsDNA contour length as functions of the drug concentration c , thermal energy and molecular extension of the dsDNA (end-to-end distance), respectively. Furthermore, we acquired reference “hat curves” via overwinding dsDNA to verify the nick-free structure of probed molecules.

All experiments were performed at 25 °C with MT buffer consisting of 10 mM phosphate buffered saline (PBS, with 137 mM NaCl + 2.7 mM KCl, pH 7.4 @ 25 °C) with 0.1 mg/ml additional bovine serum albumin (BSA, Sigma-Aldrich, Hamburg, Germany) and 0.1% TWEEN 20 (Sigma-Aldrich, Hamburg, Germany) inhibiting possible unspecific bonds. The cytostatics MTX was supplied by Baxter Oncology GmbH (Halle Westphalia, Germany), dissolved in PBS as stock solution (1 mM) and for further experiments diluted with MT buffer to concentrations from 10 nM up to 30 µM. 0.2 nM dsDNA was incubated with MTX for

2 h to reach the thermodynamic equilibrium and subsequently gently flushed into the chamber. MT force-extension experiments were performed with forces up to 10 pN after verifying the thermodynamic equilibrium binding state (data not shown, see Additional file 1). All experiments were repeated with at least 10 individual single molecules for each MTX concentration. Moreover, we replaced the complete flow cell after every statistical measurement series. The data were approximated with the WLC model and the dsDNA contour- and persistence length were fitted. In addition, by applying the transformed noncooperative McGhee-von-Hippel binding model for thermal equilibrium [36–38]:

$$\frac{\gamma}{c} = K_a \frac{\Delta x}{x_{bp}} \cdot \frac{\left(1 - \frac{n\gamma x_{bp}}{\Delta x}\right)^n}{\left(1 - \frac{(n-1)\gamma x_{bp}}{\Delta x}\right)^{n-1}} \quad (2)$$

the relation between the fractional elongation of dsDNA γ and drug concentration c was determined. K_a denotes the equilibrium constant of association for intercalation, Δx is the dsDNA elongation due to one intercalated agent molecule, x_{bp} represents the reference distance between two base pairs ($x_{bp} = 0.34$ nm). n is the binding site size per drug molecule referring to the average length of base pairs, which are responsible for the intercalation. The fractional elongation γ can be expressed as

$$\gamma = \frac{L(c) - L_0}{L_0} \quad (3)$$

where L_0 is the contour length of a bare dsDNA. The fitting errors of $L(c)$ and L_0 contribute to the uncertainty of γ , Δx , K_a and n via propagation of uncertainty. All

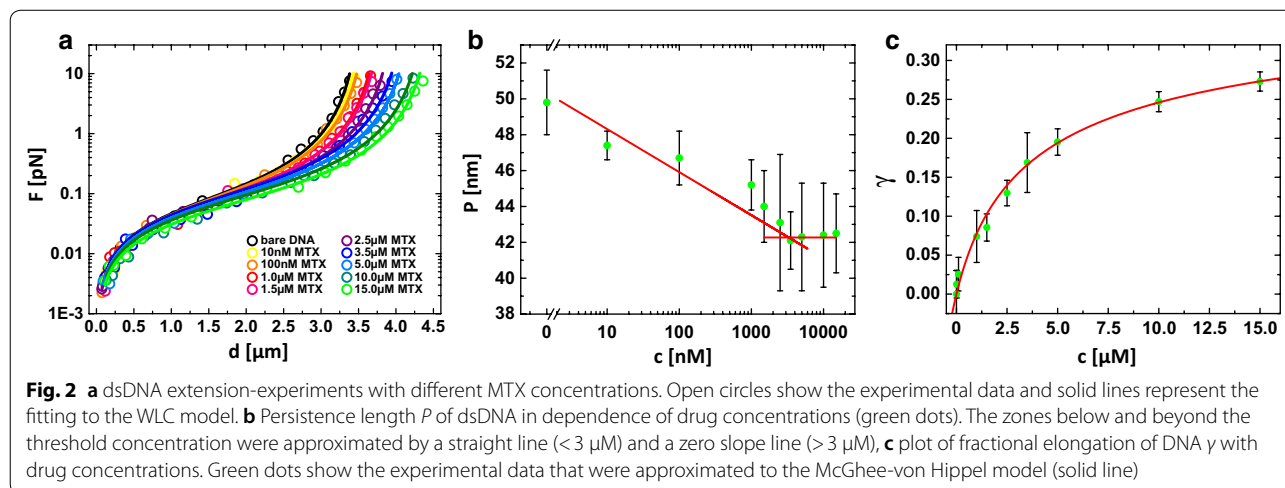
overwinding experiments were performed with a preset force of 0.2 pN where MTX was immersed with stepwise increasing concentrations.

Results and discussion

Extension-experiments

We used MT based extension and overwinding experiments to investigate the influence of the MTX association on the nanomechanical properties of dsDNA. Firstly, we conducted stretching experiments while the dsDNA remained in the torsionally relaxed state exposing its maximum end-to-end length. The force-extension curves of MTX-dsDNA mix are presented in Fig. 2a. The contour- and persistence length of the investigated dsDNA molecules were estimated via approximating the data to the WLC-model.

At low MTX concentrations up to 3 μ M, we discovered successive shifts of the force-extension curves indicating larger dsDNA contour lengths. Interestingly, at the same time the persistence length decreased from about 50 ± 2 to 42 ± 2 nm. Further increasing the drug concentration, merely an increment of the contour length was detected. At a drug concentration of 15 μ M, we found a dsDNA-elongation of 27%. In previous work, we were able to categorize the binding mode of a dsDNA-binding agent by its influence on the nanomechanical properties of the host molecule, i.e. an intercalator elongates the dsDNA virtually without affecting the bending stiffness; in contrast, a groove binder only softens the dsDNA [33]. That leads to the conclusion that MTX-dsDNA association exhibits a concentration-dependent bimodal binding mechanism. Primarily, MTX intercalates and groove-binds to dsDNA simultaneously, i.e. the planar anthraquinone ring interacts with the dsDNA base pairs in both intercalating and groove-like binding modes. Moreover,



the aminoethylamino side chains bind electrostatically to the negatively charged phosphate backbones strengthening the MTX-dsDNA interaction. This matches with the results from the earlier reports [14–19, 22, 39–41]. Beyond the threshold concentration of 3 μM , the intercalation becomes dominant. Notably, in the case of bimodal binding, it is still not clear in which groove the electrostatic interaction occurs. Lown et al. and Wang et al. suggested that two aminoethylamino chains fit to the major groove by electrochemical experiments and a high-field 1H-NMR analysis, respectively [14, 18, 20]. In contrast, Mazerski et al. reported a minor-groove association of both side chains [17]. Several other work found that the helically shaped chains of MTX can associate in both grooves. However, the interaction in the minor groove was found less favorable and sequence-selective [15, 16, 19].

Determination of binding mechanism

In addition, we approximated the fractional elongation data to the non-cooperative McGhee-von Hippel binding model (Fig. 2c) and obtained an elongation per intercalated drug molecule of $\Delta x = 0.37 \pm 0.02$ nm, corresponding to a rise of a B-DNA base pair (0.34 nm). The binding site size n was determined as $n = 2.51 \pm 0.11$ bp, which is typical for a monointercalator and conforms to the “nearest neighbor exclusion principle” [42–44]. This matches very well with previous results [18, 21, 40] although earlier Kapuscinski et al. also reported a n -value of 5 bp for MTX [39]. Analogously, we calculated an equilibrium constant of association of $K_a = (0.98 \pm 0.06) \times 10^5 \text{ M}^{-1}$, which is consistent with the results of Kapuscinski et al. of $K_a = 2.5 \times 10^5 \text{ M}^{-1}$ [21] but somewhat lower than

published by other groups [15, 18, 22–25, 39]. However, since MTX apparently presents a more complex bimodal binding mode, the theoretical model might be of a somewhat limited applicability.

Overwinding-experiments

In order to determine the unwinding angle of the MTX-intercalation, we performed overwinding-experiments that allowed us to twist individual nick-free dsDNA molecules in a well-defined manner. The pulling force was preset to 0.2 pN. The resulting supercoiling states were recorded as so called “hat curves” (Fig. 3a). At such small forces, a bare dsDNA molecule exhibits a symmetric torsional behavior. The peak positions of these curves describe the rotationally relaxed state of the dsDNA double helix. Starting from here, a hat curve can be divided into two phases (Fig. 1b, blue dashed line). In the first phase, the dsDNA length hardly changes upon twisting where the mechanical torque on dsDNA is released along the double strands. In the second phase, the dsDNA end-to-end distance decreases linearly with the number of added turns where plectonemes are formed [33, 45–47]. The buckling number N defines the crossover regime of these two phases (Fig. 1b). In contrast, a multiple rotation of a nicked dsDNA molecule causes no under- or overwinding since the single strand can rotate around the phosphodiester bond in idle state [48]. Such structural characteristics of dsDNA polymers can be used to study dsDNA unwinding induced by drug-intercalation. The local unwinding generates positive supercoilings which can be detected as a sudden dsDNA length decrement or a shift of hat curves [31, 33, 46, 49–52].

The overwinding experiments were recorded with added MTX concentrations up to 28 μM . The hat curve

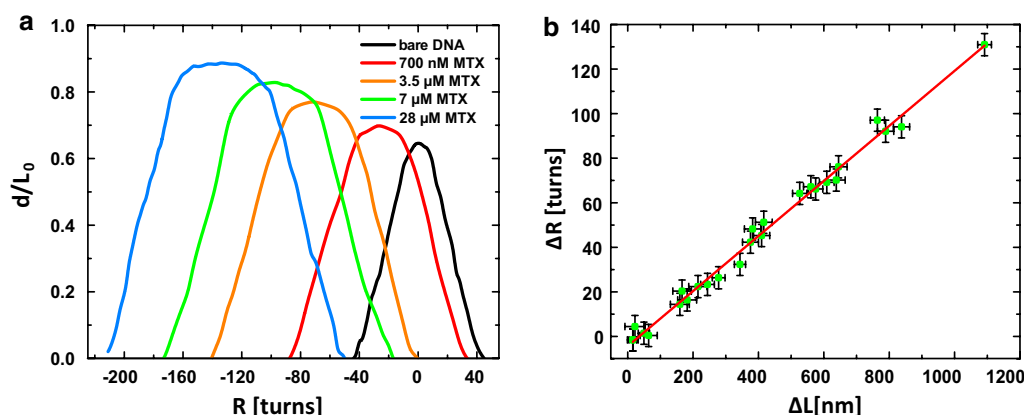


Fig. 3 **a** Results of single DNA molecule overwinding experiments with stepwise increased MTX concentration at a preset force of 0.2 pN; **b** plot of the change of the rotation number ΔR with corresponding elongation of the DNA contour length ΔL . The slope (linear approximation, solid line) allows estimating the unwinding angle per intercalated MTX molecule

of bare dsDNA was taken as reference (black curve, Fig. 3a). By increasing the MTX concentration, an obvious shift of the hat curves to negative rotation numbers was observed, indicating a DNA unwinding and further supporting the intercalative binding mode of MTX [44]. In addition, a height increment of the hat curves implies an intercalation induced dsDNA elongation that is fully consistent with our extension experiments [44].

Moreover, we evaluated and plotted the change in the rotation number ΔR and the elongation of the dsDNA contour length ΔL (Fig. 3b). The linear approximation of the data gave us a slope of 0.121 ± 0.002 turns/nm.

According to the following correlation

$$\theta = \frac{\Delta R}{\text{number of bound MTX}} = \frac{\Delta R}{\Delta L} \cdot \Delta x \quad (4)$$

the unwinding angle per intercalated MTX molecule θ can be calculated combining the slope of the linear fit and the previously determined elongation per drug molecule Δx [31, 33, 49]. As a result, we obtained an unwinding angle of 0.045 ± 0.003 turns/drug corresponding to $\theta = 16 \pm 1^\circ/\text{drug}$. This result is in full accordance with the reported value of Lown et al. from their independent viscosity and topoisomerase assays (17.5° , [15]), but considerably lower compared to the report from Kapuscinski et al. (26.5° , [39]).

Conclusion

In summary, we investigated the nanomechanical binding mechanism of MTX to dsDNA at room temperature in PBS buffer by employing a MT single molecule nanosensor. As a conventional mono-intercalator, MTX displayed a fast equilibrium assembly compared with bis-intercalators and threading intercalators [53–58]. By means of extending and overwinding individual DNA molecules, we observed an elongation, softening and untwisting of the DNA double helix upon MTX binding in a concentration dependent manner. Based on earlier findings [33], we identified a bimodal association mode, i.e. MTX exhibits simultaneously an intercalative and groove-binding behavior. In addition, we determined a threshold concentration of $3 \mu\text{M}$ at which the primary bimodal association declines and mere intercalation becomes dominant. Furthermore, we estimated a binding site size of $n \approx 2.5$ bp, which corresponds to the results of previous reports ($n = 2.6$ – 3.0 bp) [18, 21, 40]. An elongation of $\Delta x \approx 0.37$ nm induced by each drug molecule was estimated, which is typical for a mono-intercalator, since the bond between the drug molecule and DNA base pairs is stabilized through π -stacking. Moreover, we found that each

intercalated MTX molecule unwinds the native DNA helix with an angle θ of about 16° , compensating the elongation-induced tension. Finally, the equilibrium constant of association of MTX-dsDNA interaction was determined to be about $K_a \approx 1 \times 10^5 \text{ M}^{-1}$, which is significantly lower than in previous reports [15, 18, 22–25, 39]. However, other anthraquinone derivatives like DRAQ5 were found to occupy a similar binding affinity to DNA [33, 59–63]. The results of this work help to further characterize and quantify the biophysical binding mode of mitoxantrone to dsDNA and in turn support the medical regulation processes.

Additional file

Additional file 1. Force clamp experiments of dsDNA molecule with $3 \mu\text{M}$ MTX at different forces. dsDNA was incubated with $3 \mu\text{M}$ Mitoxantrone for 2 h in the relaxed state. The forces 0.1, 1, 5 and 10 pN were successively applied to the bead so that the DNA molecule was stretched. After a delay of 10 s, which was as well included in the force-extension measurements, the DNA extensions were recorded as a function of time. The constant DNA lengths in a large time scale (10 min) indicate that the mitoxantrone already equilibrated its association to the DNA before the force measurements were taken and displayed a fast equilibrium assembly. Here, d/L_0 is the normalized end-to-end distance of the DNA molecule and L_0 represents the DNA contour length in the absence of mitoxantrone.

Authors' contributions

DA, KT, MR and YW designed the study. KT and YW contributed substantially to the sample preparation. DK performed all measurements and analyzed the data. DK, YW and DA wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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