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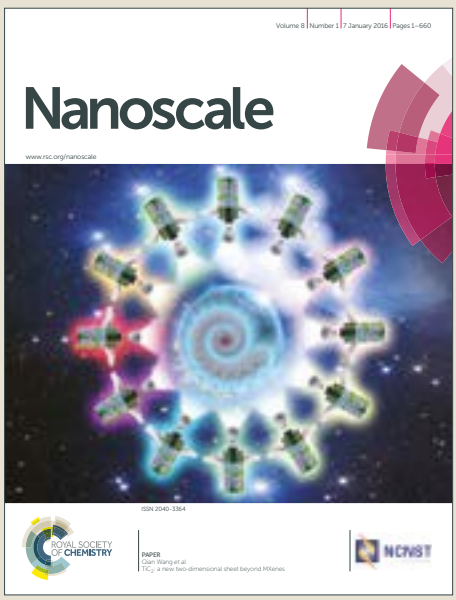

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Advantages of an Optical Nanosensor System for Mechanistic Analysis of a Novel Topoisomerase I Targeting Drug: A case study

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Abstract

The continuous need for the development of new small molecule anti-cancer drugs calls for easy accessible sensor systems for measuring the interaction between vast numbers of new drugs on their potential cellular targets. Here we demonstrate the use of an optical DNA biosensor to unravel the inhibitory mechanism of a member of a new family of small molecule human topoisomerase I inhibitors, the so-called Indeno-1,5-naphthyridines. By analysing human topoisomerase I catalysis on the biosensor in the absence or presence of added drug complemented with a few traditional assays, we demonstrate that the investigated member of Indeno-1,5-naphthyridine family inhibited human topoisomerase I activity by blocking enzyme-DNA dissociation. To our knowledge this represents the first characterized example of a small molecule drug that inhibits a post-ligation step of catalysis. The elucidation of a completely new and rather surprising drug mechanism-of-action by the use of the optical real time sensor highlights the value of this assay system in the search for new topoisomerase I targeting small molecule drugs.

1. Introduction

Due to late diagnosis, chemotherapy with small molecule drugs is often the only possible treatment of human cancers. Even though a large number of different chemotherapeutic agents exist on the market, many of them are hampered by limitations [1–3]. Hence, the continued development of new anti-cancer drugs remains a priority and consequently so does the development and validation of easy accessible sensor systems for measuring the effect of new drugs on their potential molecular targets.

One of the cellular targets in cancer chemotherapy is the enzyme human topoisomerase I (hTopI). This enzyme controls the topological state of genomic DNA using a reaction mechanism that includes the following discrete steps: non-covalent DNA binding, cleavage of one strand in the DNA duplex creating a covalent protein-DNA complex and a free 5' hydroxyl DNA end, strand rotation of the cleaved DNA strand around the uncleaved strand, ligation of the free 5´-hydroxyl DNA end, dissociation and enzyme turnover [4,5].

hTopI is the target for several small molecule inhibitors. Of these, anti-cancer drugs of the Camptothecin (CPT) family, which are currently in clinical use is one of the most important drugs[6,7]. These drugs act by selectively inhibiting the ligation step of hTopI catalysis causing accumulation of hTopI bound nicks in the genome, which ultimately results in cell death [8,9]. Despite the promising results obtained with the clinically approved CPTs, the drugs have several limitations and the development of cellular drug resistance is a frequent obstacle to treatment [10–13]. Due to these problems intensive effort is put into the development of new and more effective hTopI inhibitors.

Screening for and unravelling the mechanism of new potential small molecule chemotherapeutics is an expensive and tedious process with standard *in vitro* methods [14–18]. Therefore, with the increased accessibility of affordable synthetic DNA with various modifications, real-time DNA sensors are becoming attractive tools for drug screening and investigation of drug action. Currently molecular sensors for a large array of potential drug targets are available [19–22]. Most of these consist of one or more DNA strands coupled to various chemical

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compounds such as organic fluorophores, quenchers, chromophores, quantum dots or other nanoparticles and are designed in such a way that reaction with their target enzyme either turn-on or turn-off the reported signal. Following this principle DNA sensors that allow cleavage by endonucleases or repair enzymes or the ligation by hTopI to be followed over time have been reported [23–28]. In line with this development we recently developed an optical sensor system for quick, low-cost kinetic investigations of the combined hTopI cleavage and ligation steps of catalysis [29]. This sensor enables also the mechanism of inhibition by potential new hTopI inhibitors to be elucidated in a time-efficient manner.

In the present study we describe how the sensor was successfully used to unravel the inhibitory mechanism of a novel small molecule inhibitor of hTopI, 6-(2-naphthyl)-*7H*-indeno[2,1-*c*][1,5]naphthyridine-7-one I (compound **I**) (Fig. 1A). Using the optical sensor system we were able to obtain kinetic data describing the effect of compound **I** on hTopI catalyses and by this we demonstrated that the drug inhibits hTopI catalyses by blocking enzyme-DNA dissociation (i.e. compound **I** inhibits the catalytic off rate of hTopI). To our knowledge compound **I** represent the first characterized example of a small molecule drug that acts by inhibiting a post-ligation step of catalyses, suggesting that the indeno[1,5]naphthyridinone family represent a family of small molecule hTopI inhibitors with a completely novel mechanism of action. Indeed the elucidation of a completely new and rather surprising drug mechanism by the use of the optical real time sensor truly underscore the value of this assay system.

2. Materials and Methods

Synthetic DNA oligonucleotides:
All DNA oligonucleotides (sequences are reported in the specific method) were purchased from DNA-technology, Denmark.

Enzymes and Materials:
HTopI enzyme was expressed in the *S. cerevisiae* null strain RS190 (a kind gift from R. Sternglanz, State University of New York) and purified as previously described [30]. The protein concentrations were estimated from coomassie blue-stained SDS-polyacrylamide gels by comparison to serial dilution of BSA. Negatively supercoiled plasmid was obtained by purifying pUC18 from E. coli using midi prep kit from Qiagen.
Me_2SO (DMSO) and Camptothecin (CPT) were purchased from Sigma-Aldrich. CPT was dissolved in 99.9 % DMSO at 20 mM and stored at -20 °C.
Indeno[1,5]naphthyridinone I (Compound **I**) was synthesized as described (Fig. S1), dissolved in 99.9 % DMSO at 10 mM and stored at -20 °C.

Assay for hTopI mediated relaxation of plasmid DNA:
Relaxation reactions were carried out in the presence of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 150 mM or 50 mM NaCl. The enzyme storage buffer additionally provided 2.5 % glycerol, 0.25 mM Tris-HCl pH 7.5, and 0.025 mM EDTA to the reaction. When indicated, different concentrations of CPT and compound **I** dissolved in DMSO (2.5 % (v/v) final concentration) were

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added. Control reactions with no addition of drug were supplied with DMSO (2.5 % (v/v) final concentration).
A total of 200 fmol of pUC18 were incubated with 150 fmol of purified hTopI at 37 °C for indicated time intervals. When indicated, compound **I** was pre-incubated with purified hTopI and pUC18 at 37 °C for 10 minutes. Reactions were terminated by the addition of SDS to a final concentration of 0.2 % (w/v). Samples were subjected to proteolytic digestion by 1 mg/ml proteinase K at 37 °C for 1 hour prior separation of relaxation products in a 1 % agarose gel, run in TBE (48 mM Tris, 45.5 mM Boric Acid, 1mM EDTA). After the run, DNA bands were visualized by staining of the gel with 0.5 μg/ml ethidium bromide and analysed using the Bio-Rad Gel XR+ System.

hTopI mediated cleavage/religation equilibrium:

Oligonucleotide AS22 (5'-TTG CTT AAG CTT TTT TAA TGG CAT CAG AAA TTT GGA TCC GCT ATC ATA TGA ATT ATA GCG AAT TCG-3') that contains a hTopI high affinity cleavage site, was 5´-end labelled with [γ^{32}P] ATP by employing the T4 polynucleotide kinase reaction using [γ-32P]ATP as phosphoryl donor. Unreacted [γ-32P]ATP was removed by spin dialysis on a G-50 column (GE-healthcare). The AS57 complementary strand (5'-CGA ATT CGC TAT AAT TCA TAT GAT AGC GGA TCC AAA TTT CTG ATG CCA TTA AAA AAA GCT TAA GCA A-3') was 5´-end phosphorylated with unlabelled ATP. The two strands were annealed with a 2-fold molar excess of AS57 to form the equilibrium substrate. DMSO, 80 μM CPT or 80 μM Compound **I** were pre-incubated with 660 fmol of hTopI 15 min at 37 °C. A final concentration of 20 nM duplex equilibrium substrate was added and the reactions incubated at 37 °C for 10 minutes in a reaction buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 100 mM NaCl. The reactions were stopped by adding 0.7% SDS and precipitated by the addition of 300 mM NaCl and 3 volumes of 96 % Ethanol. The precipitated samples were digested with 5μl of 1mg/ml Trypsin for 30 min at 37 °C and mixed with an appropriate volume of loading buffer [80% (v/v) deionized formamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) Xylene xyanol] prior to loading on a 12% denaturing urea/polyacrylamide gel and run in TBE (48 mM Tris, 45.5 mM Boric Acid, 1mM EDTA) for analysis. The gel was dried and the radiolabelled DNA bands were visualized by PhosphorImager (BIORAD).

Sensor based experiment:

The hTopI DNA sensor consists of two oligonucleotides the L-oligo: 5'-AGA AAA ATT TTT AGA GGC CTA GC-C6amine and the C-oligo: 5'-GCT AGG CCT GTA AAA ATT T**T**T CTA AGT CTT TTA GAT CAT CGT TAT TCG ATG ATC TAA AAG ACT **TA**G A-BHQ1. The bold underlined T was labeled with a 6-carboxyfluorescein (FAM), the bold underlined TA indicates the hTopI cleavage site, and the Black Hole Quencher 1 (BHQ1) was attached through a phosphorthioate bond. The two oligoes were hybridized by incubating 20 μM L-oligo with 10 μM C-oligo in 10 mM Tris-HCl pH 7.5, 1 mM EDTA at 95 °C for 5 minutes on a metal block following 10 minutes cool down at room temperature. When indicated the L-oligo was 5'-end phosphorylated prior to the annealing.

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Activity measurements were carried out using 0 μM, 0.0625 μM, 0.125 μM, 0.25 μM, 0.5 μM, or 1 μM of the sensor in the presence of 80 μM compound **I** dissolved in DMSO (2 % (v/v) final concentration) and 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 24 mM NaCl provided by the hTopI storage buffer, in a final volume of 25 μL. Note, that the lowest concentration of substrate was chosen sufficiently high to ensure surplus of substrate relative to active enzyme in the duration of the experiment resulting in a reaction that follow Michaelis-Menten kinetics. Control samples were supplied with DMSO (2 % (v/v) final concentration). The components were mixed in two separate tubes, one containing the substrate and the second containing the hTopI and compound **I**. The enzyme was pre-incubated with the drug at 37 °C for 10 minutes and the tubes were mixed immediately before the first measurement took place. Development of fluorescence was followed in a Mx3000P qPCR machine (Agilent Technologies, Inc.) and data were collected every 15 seconds for the first 20 minutes and every 1 minute for the last 40 minutes. Raw data were plotted as fluorescence as function of time and the slopes of the curves in the linear interval between 30 and 60 minutes were plotted as function of DNA sensor concentration. The experiment was made in 8 repetitions and the non-linear Michaelis-Menten fits were performed using the GraphPad prism software. Km and Vmax values were calculated from the fits by the software.

Measure of hTopI Off-rate:

Purified hTopI enzyme was mixed with 100 μM compound **I** dissolved in DMSO or with DMSO alone in a reaction buffer of 10 mM Tris-HCl pH 7.5, 40 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.1 mM DTT, ~10% glycerol w/v in 9 μL. The DMSO constituted maximally 1.1% of the total volume. Samples were incubated for 10 min. at 37°C to allow the drug to interact with the enzyme. Then 1 μL of either 25 μM linear DNA fraction (made of sense oligonucleotide: 5'-ATT TTT CTA AGT CTT TTA GAT CGA ACG ACT CAG AAT G-3' and antisense oligonucleotide: 5'-ACT ACC ATT CTG AGT CGT TCG ATC TAA AAG ACT TAG A-3') or 375 nM circular plasmid DNA (pUC18 plasmid) was added to the samples, respectively, and samples were incubated again for 10 min. at 37 °C to allow for interaction between enzyme, drug and DNA. Finally, the 10 μL sample containing the enzyme with or without compound **I** and with linear or circular DNA were quickly moved to new pre warmed tubed containing 15μL of the optical sensor in similar reaction buffer (10 mM Tris-HCl pH 7.5, 30 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.1 mM DTT, ~10% glycerol w/v) using a multi-pipetter and the fluorescence was measured every 5 sec for 20 minutes. The final reaction volume was 25 μL and the final concentration of the optical sensor was 1 μM. The final concentration of base pairs of the linear DNA fragment, the circular plasmid DNA and the optical sensor was similar (~35-45 μM). The final drug concentration was 40 μM.

As the sensor was mixed with the enzyme, drug and DNA samples, the fluorescence drifted slightly due to interaction with the added DNA. In order to avoid this drifting, the fluorescence of the negative sample, where no enzyme had been added, was subtracted from the fluorescence of the positive samples. The fluorescence was then plotted starting from zero in GraphPad Prism software.

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3. Results and discussion

*Compound **I** synthesis:*

The preparation of 6-(2-naphthyl)-*7H*-indeno[2,1-*c*][1,5]naphthyridine-7-one **I** (Fig. 1A) has been performed following procedures as previously reported [31]. Briefly, the synthesis of compound **I** was based on the Povarov type [4+2]-cycloaddition reaction [32,33] and involves a multicomponent reaction of 3-aminopyridine, 2-naphthaldehyde and indene [34,35], followed by dehydrogenation and subsequent methylene oxidation reaction (Fig. S1).

*Compound **I** inhibition of hTopI relaxation:*

As part of the continued search for new hTopI targeting anti-cancer drugs it was recently demonstrated that members of the novel family of hTopI inhibitors, the indeno[1,5]naphthyridinone drug family, inhibited hTopI mediated DNA relaxation. This inhibition was strongly enhanced by pre-incubation of the drug with the enzyme prior to addition of the supercoiled DNA substrate [31].

These results were confirmed in our investigations using standard time course relaxation experiments. When the indeno[1,5]naphthyridinone drug family member, compound **I** (Fig. 1A) was added simultaneously with a supercoiled DNA substrate TopI mediated relaxation activity was slightly inhibited (Fig. 1B, compare lanes 4-6 with lanes 1-3). Similar results were obtained using another member, compound II, of the indeno[1,5]naphthyridinone drug family (Fig. S3). However, since compound I appeared slightly more potent that compound II and III in several assay repetitions, we decided to continue our investigations using compound I as a representative of the drug family. In contrast, the DNA relaxation was almost completely inhibited when compound **I** was pre-incubated with hTopI before addition of the supercoiled DNA (Fig. 1B, compare lanes 7-9 with lanes 1-3). Compared to the well-known hTopI inhibitor CPT, compound **I** was approximately 6 times less potent as an inhibitor of hTopI when incubated at similar conditions, i.e. without pre-incubation of drug and enzyme, as demonstrated by titration experiments (Fig. S2) and relatively high concentrations of the drug were necessary to obtain an inhibition at these conditions. We therefore chose to continue our studies using a concentration of compound **I** of 80 μM, which is the highest possible concentration that could be obtained in the reactions described below using the available stock.

*Effect of compound **I** on hTopI Mediated Cleavage of the Sensor:*

The available evidence demonstrates that most small molecule inhibitors for which the effect are enhanced by pre-incubation with the enzyme inhibit the DNA binding and/or cleavage step of hTopI catalysis [36–38]. To address this possibility for compound **I** we took advantages of an optical sensor designed for real-time measurement of the cleavage-ligation kinetics of hTopI [29]. The structure of this sensor is schematically represented in Fig. 2. As depicted, the sensor is composed of two oligonucleotides (denoted the C- and the L-oligo, respectively), which hybridize to form a nicked hairpin structure with a highly preferred hTopI cleavage site as indicated by the arrow [29]. Indeed numerous

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investigations of hTopI interaction with the utilized DNA sequence have unambiguously shown cleavage at the indicated position (Andersen et al, PMID: 2987828 REF). Upon cleavage (of the C-oligo), the generated tri-nucleotide (AGA) with the quencher quickly diffuses away resulting in an increase of fluorescence emission that can be measured in real-time using a fluorescence reader. Subsequent to cleavage, the break is sealed by ligation of the free 5´-OH end of the L-oligo and hTopI is released to engage in a new catalytic cycle. The presented assay builds upon the same principles as traditional assays for measurement of hTopI cleavage-ligation activities [14,15,39] but distinguish itself by the ease of which large amount of data can be collected. At least using purified enzyme preparations it is specific for the cleavage-ligation of hTopI and can for instance not react with a reminiscent enzyme activity such as T4 DNA ligase (Fig. S4. Moreover, the sensor provides a valuable tool for comprehensive kinetic analyses of hTopI cleavage-ligation as previously described [29].

First we addressed the effect of compound **I** of hTopI mediated cleavage on the DNA sensor. As previously described the cleavage rate on the DNA sensor can in principle be obtained by analysing the reaction rates within the first minutes subsequent to addition of enzyme with or without drug [29]. During this time interval a steep increase in signal is detected as a function of time. This part of the reaction is termed the burst phase, which (as described in [29]) primarily can be ascribed to the pre-steady state cleavage of the sensor. However, it cannot be excluded that the reaction constant (k_2, Fig. S5) of the steady phase (which is observed after the burst phase) may have some influence on the reaction constants of the burst phase (k_{burst}, Fig. S5). Hence, the rate of the burst phase is only an approximation of the rate of cleavage [29]. In order to measure the cleavage rate on the sensor precisely we, therefore, investigated if it was possible to prevent ligation and enzyme turnover by 5´-end phosphorylation of the L-oligo. This would make it possible to measure the initial cleavage step of catalyses only. As evident from Fig. 3A, measuring hTopI activity on the modified sensor (termed the 5'-phospho-sensor in the following) resulted in a reaction curve with a burst phase that flattens out directly without showing any evidence of the steady-state phase, which typically follows the burst phase on the unmodified sensor (Fig. S5A). This strongly suggests that only the first cleavage reaction of catalysis was completed. The addition of 80 μM compound **I** did not change the reaction of hTopI on the 5'-phospho-sensor when compared to the reaction without addition of drug (Fig. 3B). This result, which was confirmed using traditional hTopI cleavage assays as described in [14,15,18,38] (Fig. S6 A, Fig S6 B) demonstrated that the drug did not affect hTopI mediated cleavage. Hence, the effect of compound **I** on the reaction measured when using the un-phosphorylated sensor (see below) can only result from inhibition of catalytic post-cleavage steps e.g. ligation or enzyme turnover.

*Effect of compound **I** on Steady Phase Reaction of hTopI on the Sensor:*

To further investigate the mechanism of compound **I** inhibition of hTopI, the steady phase reaction (with or without drug) rates were measured on the sensor in time course experiments. For this purpose the enzyme was incubated in the absence or presence of drug with different concentrations of un-phosphorylated sensor ranging from 0.0625-2 μM. These sensor concentrations gave rise to a molar surplus of substrate compared to hTopI to ensure Michaelis-Menten

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conditions during the time where data were acquired. To calculate the kinetic parameters of the steady phase of two reactions with or without compound **I**, linear least square fits were made from signal measurements obtained in the time interval 30-60 min. and the slopes were plotted as a function of the DNA sensor concentration present in the sample (Fig. 4B). The apparent maximal velocity (V_{max}) and K_M values (see Table 1) were found by fitting the points in Fig. 4B to the Michaelis-Menten model (Fig. S5B) using GraphPad Prism-software. As evident from the plot shown in Fig. 4B and the calculated values (Table 1) compound **I** inhibited the steady phase of the reaction indicating an inhibitory effect of the post-cleavage steps of the reaction. Moreover, addition of compound **I** to the reaction did not change K_M significantly, while V_{max} was reduced compared to the control reaction without added drug. This is consistent with a non-competitive inhibition signature of compound **I** where the drug can bind to the enzyme both with and without the presence of DNA and is in agreement with the increased inhibitory effect of compound **I** upon pre-incubation with hTopI (Fig. 1) [31]. Note, the calculated K_M is lower than what was previously found for hTopI on the utilized sensor (K_M: 0.47 ±0.2) [29]. The reason for this difference is not clear, but may relate to the presence of DMSO in the current Michaelis-Menten study or to a slightly lower NaCl contribution from the purified enzyme fraction used in these experiments.

*The ability of compound **I** to Induce Accumulated hTopI Cleavage Complexes on Double Stranded DNA:*

The catalytic step that succeeds cleavage on the optical sensor is DNA ligation. Indeed selective inhibition of this step of catalysis has previously been reported for CPT, we therefore set up to investigate if this reaction step was affected by compound **I**. Ligation cannot easily be addressed directly on the optical sensor. Hence, to address this catalytic step the optical sensor system was complemented by traditional assays for investigation of hTopI cleavage-ligation. A well-known effect of the selective inhibition of hTopI mediated DNA ligation by CPT is the accumulation of enzyme bound nicks (cleavage complexes) in double stranded DNA. If compound **I** acted by inhibiting hTopI ligation a similar phenotype should be observed for this drug. This possibility was tested in a classical equilibrium assay by using a radiolabelled double stranded DNA fragment as a substrate for hTopI catalysis (Fig. 5). In this assay accumulated cleavage products would appear as products with a faster gel-electrophoretic mobility than the substrate after protease removal of covalently attached hTopI. Parallel assaying of the CPT effect in this assay was used as a control. As expected the addition of CPT resulted in the induction of cleavage products (Fig. 5, lane 3, C1 and C2) that were not observed without drug addition (Fig. 5, lane 2). In contrast, compound **I** did not lead to accumulation of cleavage products in the double stranded fragment (compare lanes 2 and 4 of Fig. 5). Likewise, accumulation of hTopI induced nicks in plasmid DNA was only observed in the presence of CPT and not upon addition of compound **I** (data not shown). Hence, the observed inhibition of relaxation by compound **I** can most likely be ascribed to the drug affecting reaction steps that succeeds ligation e.g. enzyme dissociation and turnover.

Surprisingly, when using the so-called suicide system (in which the ability of preformed cleavage complexes to ligate an externally added DNA

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oligonucleotide is measured) compound **I** exhibited an inhibition profile similar to that of CPT (Fig. S6). This experiment was performed in parallel with the above described cleavage-ligation equilibrium assays performed on a double stranded DNA fragment (Fig. 5) or a plasmid (data not shown) and, hence, the lack of accumulated cleavage complexes observed in these experiments was not due to inactivity of the utilized batch of compound **I**. Rather, as will be discussed later the different results may be caused by the differences inherent to the two assay setups. In the equilibrium experiments it is the 5´-OH end that is generated during cleavage that is ligated and, hence, the nucleophile to be ligated will be more or less perfectly position in the catalytic of hTopI as soon as it is created. In the suicide setup the ligator DNA with a 5'-OH end is added after cleavage and covalent attachment of the enzyme and, hence, the 5'-OH DNA end has to enter the cleavage complex before ligation can be performed. Taken together, the results of the equilibrium experiments and the ligation experiment performed using the suicide substrate system suggests that whereas CPT inhibits ligation *per se*, compound **I** affect hTopI in a manner that does affect ligation on naturally double stranded DNA but rather prevent access of an externally added ligator DNA to the catalytic pocket of a preformed cleavage complex.

Elucidation of the compound ***I*** *Mechanism of Action*

To investigate the effect of compound **I** on the catalytic steps of hTopI catalysis that succeeds cleavage-ligation on natural double stranded DNA, *i.e.* substrate dissociation and enzyme turnover a competition experiment was performed using either linear or circular unlabelled double stranded DNA as a competitor for cleavage of the 5'-phospho-sensor. The rationale of this experimental setup relies on the mechanism of DNA binding of hTopI that results from the overall bi-lobed clamp structure of the enzyme [5,40]. Upon DNA binding the enzyme clamp closes around the DNA helix in a manner that is controlled by a flexible hinge region and the so-called lips that are brought in close proximity to each other [5,40,41]. Inherent to this mechanism of binding, dissociation from a linear DNA substrate may occur without major conformational changes and without clamp opening *per se*, since the enzyme may simply slide off the DNA substrate. Dissociation from a circular DNA substrate on the other hand would require larger conformational changes, e.g. clamp opening (see the schematic representation of the assay in Fig. 6A and Fig. B, left panels) [40,41].

The effect of compound **I** on the ability of hTopI to dissociate from either the linear or circular DNA competitor and cleave the 5´-phospho-sensor was measured by pre-incubating the enzyme with each of the DNA competitors in the absence or presence of drug before the sensor was added and cleavage followed over time. As evident from Fig. 6A, pre-incubation of hTopI with circular DNA competitor and compound **I** completely abolished cleavage of the 5'-phospho-sensor (note, that this inhibition profile did not depend on the super helical state of the circular DNA competitor and similar results were obtained using supercoiled or relaxed DNA circles (data not shown)). Without addition of the drug the enzyme was capable to dissociate from the circular competitor DNA and react with the DNA sensor.

In contrast to the results obtained by using circular competitor DNA, pre-incubation of the enzyme with the linear competitor DNA and compound **I** resulted in an only modest inhibition of 5'-phospho-sensor cleavage (Fig. 6B).

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The most obvious difference between the linear and circular competitor DNA is the ease by which hTopI in the closed clamp conformation may be envisioned to dissociate from the substrate. The complete inhibition of cleavage observed upon pre-incubation of hTopI with compound **I** and circular competitor DNA (but not linear competitor DNA) suggest that the drug may act by preventing the relay of conformational changes necessary to open the protein clamp and release circular DNA [42–44]. This is in line with the non-competitor inhibition profile of compound **I** (Fig. 4B) and the observed inhibition of relaxation. Indeed, the ability of the closed clamp conformation of hTopI to inhibit DNA relaxation was previously demonstrated in experiments were the clamp was covalently closed by cysteine bridges between cysteines in the upper and lower lobes of the protein [45]. Moreover, freezing of the hTopI clamp in a rather closed conformation may prevent access of an externally added ligator strand to preformed cleavage complexes and thereby explain the observed inhibition of ligation by compound **I** when using the suicide-substrate setup (Fig. S6) but not on double stranded DNA (Fig. 5).

4. Conclusions

The described study demonstrates the advantages of using a fluorophore and quencher coupled DNA sensor for real time studies of hTopI catalyses to unravel important mechanistic aspects of a novel hTopI directed small molecule drug. Traditionally such studies include *in vitro* investigations involving cell cultures combined with various cell free systems that is time consuming and labour-intensive and requires specialized equipment. Here we demonstrate how such assays may be complemented or even replaced by the easy to use and fast real-time DNA sensor for kinetic analyses of hTopI catalyses.

The drug under investigation, 6-(2-naphthyl)-7*H*-indeno[2,1-c][1,5]naphthyridine-7-one I (compound **I**) in the presented case story represents a novel class of hTopI inhibitors (indeno[1,5]naphthyridinone I), which prevent hTopI mediated DNA relaxation using a completely novel mechanism of action. Exploiting the unique features of the DNA sensor, allowed us to demonstrate with a minimal number of experiments and time that compound **I** acts as a non-competitive inhibitor and slows down the dissociation of the enzyme from its substrate most probably by inhibiting opening of the bi-lobed clamp structure of hTopI necessary to release DNA.

It is yet unexploited how compound **I** interacts with hTopI and a direct investigation of drug binding is out of the scope of the current investigation.

However, based on the heterocyclic ring structure of compound **I** that resembles the planar structures of the hTopI specific drugs CPTs, indolocarazoles, and indenoisoquinolines(IQN)[46] it seems likely that compound **I**, like these drugs, form stacking interactions with the DNA bases upstream and downstream to the cleavage site. The scaffold of compound **I** is very similar to that of IQN, and the carbonyl group on the five-carbon-ring and the nitrogen acceptor on the opposite site of the molecule can be compared with similar groups present also in the CPT and IQN scaffold that usually interact with TopI residues ASN722 and ARG364, respectively [47,48] It may therefore be expected that compound **I** is able to dock into the major groove binding pocket formed by topI residues Glu356, Asn352, Pro431, Lys751, and Asn722[46] .

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The "Naphtyl" group in compound **I** is in a position in which other IQN inhibitors presents hydrophobic substituents that usually protrude in a pocket at the interface between hTopI and the broken DNA strand. However, the rigidity and bulkiness of this substituent in compound **I** may account for subtle, yet mechanistically determinant differences in the interaction mode compared to other hTopI drugs. Molecular dynamic simulations indicate that there is a direct motion correlation between residues within the active site pocket (including amino acids LYS532, ARG634, GLY717, THR718, THR729) and the linker domain of hTopI[43,49,50,51–55]. In particular, mutation of THR718 affected the activity of hTopI in plasmid relaxation, whilst leaving cleavage/ligation activity on oligonucleotide substrates largely unaffected[54]. Based on this, it may be speculated that the specific interactions between compound **I** and residues in the hTopI active site can affect distal structural rearrangement in the protein clamp. Such rearrangement may include clamp opening/closing, and are likely to be essential for the ability of hTopI to carry out consecutive rounds of catalysis on circular DNA.

Compound **I** is to our knowledge the first hTopI inhibitor demonstrated to selectively inhibit the enzyme-DNA dissociation step of catalysis. As such the drug may gain interest as a regulator of gene transcription without causing DNA fragmentation or it may find use in investigations of non-covalent genome interactions of hTopI that, under normal circumstances, are often too transient to be addressed. The relative ease by which the mechanism action of a drug with such a rather surprising inhibition profile was elucidated by the use of the real time sensor underline the potential power of the sensor for future investigations of novel hTopI inhibitors.

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Statement of contribution

All authors contributed extensively to the work presented in this paper.
BRK conceived the study, designed or co-designed all experiments presented and wrote the major part of the manuscript assisted by CT, RF, ELK, YPH, AC and MS. MG, CA, GR and FP synthesized compound **I** and made Fig. S1. MG performed the experiments shown in Figs. 1 and S2, S3. MG and MBA performed Fig. S6. CT

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performed Fig. S4. MBA, and CT performed most of the experiments presented in Figs. 3, 4, and 5 assisted by ELK. MBA and ELK performed the experiments presented in in Fig. 6 assisted by CT. ELK made Fig. 2, and Fig. S5.

References

1 L. Gatti and F. Zunino, *Methods Mol. Med.*, 2005, **111**, 127–48.
2 K. Masui, B. Gini, J. Wykosky, C. Zanca, P. S. Mischel, F. B. Furnari and W. K. Cavenee, *Carcinogenesis*, 2013, **34**, 725–38.
3 P. E. Lønning, *Mol. Oncol.*, 2010, **4**, 284–300.
4 J. J. Champoux, *Annu. Rev. Biochem.*, 2001, **70**, 369–413.
5 L. Stewart, *Science (80-.).*, 1998, **279**, 1534–1541.
6 J. A. Holden, *Curr. Med. Chem. Anticancer. Agents*, 2001, **1**, 1–25.
7 Y. Pommier, *Nat. Rev. Cancer*, 2006, **6**, 789–802.
8 Y. Pommier, *Chem. Rev.*, 2009, **109**, 2894–2902.
9 Y. Pommier, E. Leo, H. Zhang and C. Marchand, *Chem. Biol.*, 2010, **17**, 421–33.
10 G. L. Beretta, L. Gatti, P. Perego and N. Zaffaroni, *Curr. Med. Chem.*, 2013, **20**, 1541–65.
11 N. F. Jensen, K. Agama, A. Roy, D. H. Smith, T. D. Pfister, M. U. Rømer, H.-L. Zhang, J. H. Doroshow, B. R. Knudsen, J. Stenvang, N. Brünner and Y. Pommier, *J. Exp. Clin. Cancer Res.*, 2016, **35**, 56.
12 J. E. Chrencik, B. L. Staker, A. B. Burgin, P. Pourquier, Y. Pommier, L. Stewart and M. R. Redinbo, *J. Mol. Biol.*, 2004, **339**, 773–784.
13 A. Roy, C. Tesauro, R. Frøhlich, M. S. Hede, M. J. Nielsen, E. Kjeldsen, B. Bonven, M. Stougaard, I. Gromova and B. R. Knudsen, *PLoS One*, 2014, **9**, e99628.
14 S. Castelli, A. Coletta, I. D'Annessa, P. Fiorani, C. Tesauro and A. Desideri, *Biol. Chem.*, 2012, **393**, 1327–40.
15 R. F. Frøhlich, F. F. Andersen, O. Westergaard, A. H. Andersen and B. R. Knudsen, *J. Mol. Biol.*, 2004, **336**, 93–103.
16 J. L. Nitiss, E. Soans, A. Rogojina, A. SEth and M. Mishina, in *Current protocols in pharmacology*, 2012, pp. 789–802.
17 C. Tesauro, B. Morozzo Della Rocca, A. Ottaviani, A. Coletta, L. Zuccaro, B. Arnò, I. D'Annessa, P. Fiorani and A. Desideri, *Mol. Cancer*, 2013, **12**, 100.
18 Z. Yang and J. J. Champoux, *J. Biol. Chem.*, 2002, **277**, 30815–23.
19 H. Ilkhani, T. Hughes, J. Li, C. J. Zhong and M. Hepel, *Biosens. Bioelectron.*, 2016, **80**, 257–264.
20 D. Kahanda, G. Chakrabarti, M. A. Mcwilliams, D. A. Boothman and J. D. Slinker, *Biosens. Bioelectron.*, 2016, **80**, 647–653.
21 A. D. R. Pontinha, S. M. A. Jorge, A.-M. Chiorcea Paquim, V. C. Diculescu and A. M. Oliveira-Brett, *Phys. Chem. Chem. Phys.*, 2011, **13**, 5227–34.
22 L. Zuccaro, C. Tesauro, T. Kurkina, P. Fiorani, H. K. Yu, B. R. Knudsen, K. Kern, A. Desideri and K. Balasubramanian, *ACS Nano*, 2015, **9**, 11166–11176.
23 J. Deng, Y. Jin, L. Wang, G. Chen and C. Zhang, *Biosens. Bioelectron.*, 2012, **34**, 144–50.
24 B. Doughty, S. W. Kazer and K. B. Eisenthal, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 19979–84.
25 P. W. Jensen, M. Falconi, E. L. Kristoffersen, A. T. Simonsen, J. B. Cifuentes,

View Article Online
DOI: 10.1039/C6NR06848K

L. B. Marcussen, R. Frøhlich, J. Vagner, C. Harmsen, S. Juul, Y.-P. Ho, M. a Withers, J. R. Lupski, J. Koch, A. Desideri, B. R. Knudsen and M. Stougaard, *Biosens. Bioelectron.*, 2013, **48**, 230–7.

26 M. L. Jepsen, A. Ottaviani, B. R. Knudsen and Y.-P. Ho, *RSC Adv.*, 2014, **4**, 2491–2494.

27 Y. Qian, Y. Zhang, L. Lu and Y. Cai, *Biosens. Bioelectron.*, 2014, **51**, 408–412.

28 D. Svilar, C. Vens and R. W. Sobol, *J. Vis. Exp.*, 2012, e4168.

29 E. L. Kristoffersen, L. A. Jørgensen, O. Franch, M. Etzerodt, R. Frøhlich, L. Bjergbæk, M. Stougaard, Y.-P. Ho and B. R. Knudsen, *Nanoscale*, 2015, **7**, 9825–9834.

30 B. R. Knudsen, T. Straub and F. Boege, *J. Chromatogr. B Biomed. Sci. Appl.*, 1996, **684**, 307–321.

31 C. Alonso, M. Fuertes, M. González, G. Rubiales, C. Tesauro, B. R. Knudsen and F. Palacios, *Eur. J. Med. Chem.*, 2016, **115**, 179–190.

32 J. S. B. Forero, J. Jones and F. M. da Silva, *Curr. Org. Synth.*, 2016, **13**, 157–175.

33 L. S. Povarov, *Russ. Chem. Rev.*, 1967, **36**, 656.

34 F. Palacios, C. Alonso, A. Arrieta, F. P. Cossío, J. M. Ezpeleta, M. Fuertes and G. Rubiales, *European J. Org. Chem.*, 2010, **2010**, 2091–2099.

35 F. Palacios, C. Alonso, M. Fuertes, J. M. Ezpeleta and G. Rubiales, *European J. Org. Chem.*, 2011, **2011**, 4318–4326.

36 L.-H. Meng, Z.-Y. Liao and Y. Pommier, *Curr. Top. Med. Chem.*, 2003, **3**, 305–20.

37 Y. Pommier, *ACS Chem. Biol.*, 2013, 8, 82–95.

38 C. Tesauro, P. Fiorani, I. D'Annessa, G. Chillemi, G. Turchi and A. Desideri, *Biochem. J.*, 2010, **425**, 531–539.

39 L. Stewart, G. C. Ireton and J. J. Champoux, *J. Biol. Chem.*, 1999, **274**, 32950–60.

40 M. R. Redinbo, *Science (80-.).*, 1998, **279**, 1504–1513.

41 M. R. Redinbo, J. J. Champoux and W. G. Hol, *Biochemistry*, 2000, **39**, 6832–40.

42 G. Chillemi, P. Fiorani, P. Benedetti and A. Desideri, *Nucleic Acids Res.*, 2003, 31, 1525–1535.

43 I. D'Annessa, A. Coletta, T. Sutthibutpong, J. Mitchell, G. Chillemi, S. Harris and A. Desideri, *Nucleic Acids Res.*, 2014, **42**, 9304–9312.

44 C. M. Wright, M. van der Merwe, A. H. DeBrot and M.-A. Bjornsti, *J. Biol. Chem.*, 2015, **290**, 12068–78.

45 M. H. Woo, C. Losasso, H. Guo, L. Pattarello, P. Benedetti and M.-A. Bjornsti, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 13767–72.

46 B. L. Staker, M. D. Feese, M. Cushman, Y. Pommier, David Zembower, A. Lance Stewart and A. B. Burgin, 2005.

47 A. Coletta, S. Castelli, G. Chillemi, N. Sanna, M. Cushman, Y. Pommier and A. Desideri, *PLoS One*, 2013, **8**, e73881.

48 A. Coletta and A. Desideri, *Nucleic Acids Res.*, 2013, **41**, 9977–86.

49 Z. Wang, I. D'Annessa, C. Tesauro, S. Croce, A. Ottaviani, P. Fiorani and A. Desideri, *Biochim. Biophys. Acta - Proteins Proteomics*, 2015, **1854**.

50 I. D'Annessa, C. Tesauro, Z. Wang, B. Arnò, L. Zuccaro, P. Fiorani and A. Desideri, *Biochim. Biophys. Acta*, 2013, **1834**, 2712–2721.

51 Z. Wang, I. D'Annessa, C. Tesauro, S. Croce, A. Ottaviani, P. Fiorani and A.

View Article Online
DOI: 10.1039/C6NR06848K

Desideri, *Biochim. Biophys. Acta - Proteins Proteomics*, 2015, **1854**, 860–868.

52 G. Mancini, I. D'Annessa, A. Coletta, N. Sanna, G. Chillemi and A. Desideri, *PLoS One*, 2010, **5**, e10934.

53 G. Chillemi, I. D'Annessa, P. Fiorani, C. Losasso, P. Benedetti and A. Desideri, *Nucleic Acids Res.*, 2008, **36**, 5645–51.

54 G. Chillemi, P. Fiorani, S. Castelli, A. Bruselles, P. Benedetti and A. Desideri, *Nucleic Acids Res.*, 2005, **33**, 3339–50.

55 I. D'Annessa, C. Tesauro, Z. Wang, B. Arnò, L. Zuccaro, P. Fiorani and A. Desideri, *Biochim. Biophys. Acta - Proteins Proteomics*, 2013, **1834**, 2712–2721.

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Figures

Fig. 1 (A) Chemical structure of compound **I**; (B) Relaxation of plasmid DNA pUC18 by purified hTopI when incubated for 0.5, 1 or 5 min. at 37°C C in the presence of (2.5 % w/v) DMSO (lanes 1-3) or 80 μM compound **I** dissolved in DMSO (lanes 4-9). Lanes 4-6 show the result obtained when the DNA and drug were mixed immediately prior to addition of enzyme. Lane 7-9 is the same as lanes 4-6, except that the enzyme was pre-incubated with compound **I** for 10 min. at 37° C before addition of plasmid DNA. Lane 10 is a negative control without added enzyme. Reaction products were analysed in a 1% agarose gel and visualized by subsequent EtBr staining.

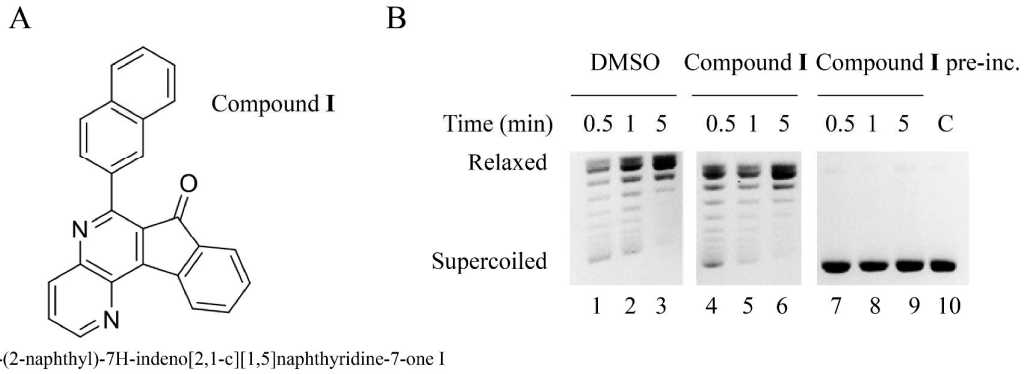

Fig. 1

Fig. 2 Structure of the DNA sensor composed of two oligonucleotides “C-oligo” and “L-oligo”. F and Q represent the fluorophore (FAM) and the Black Hole Quencher 1 (BQH1), respectively. The hTopI cleavage site is indicated by an arrow. hTopI is illustrated as a blue packman, the green arrow represent the fluorescence emitted when the quencher is removed.

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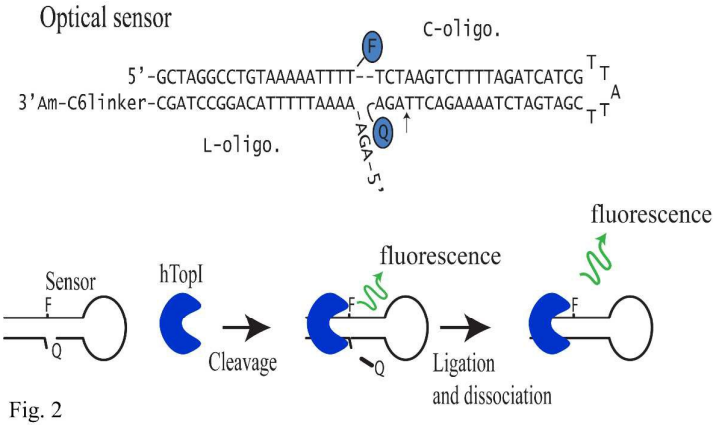

Fig. 2

Fig. 3 (A) Average of primary data obtained by measuring the amount of fluorescent signal generated when incubating a DNA sensor with an un-phosphorylated L-oligo (black curve) or a phosphorylated L-oligo (red curve); (B) Result of assaying hTopI activity using the 5'end-phosphorylated DNA sensor (L-oligo) with DMSO or 80 μM compound **I**.

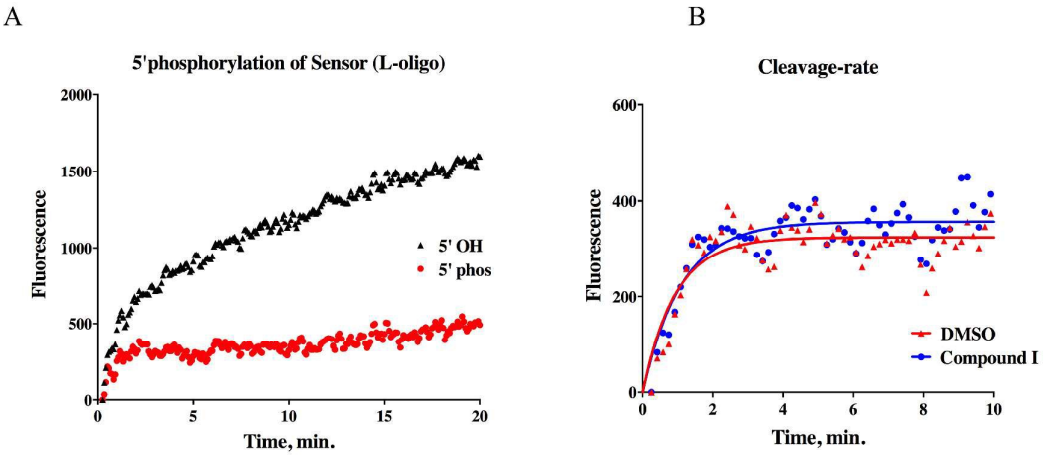

Fig. 3

Fig. 4. Investigation of steady phase reactions on the sensor with or without compound I. (A) Average of primary data obtained when hTopI was incubated with DMSO (left panel) or 80 μM compound **I** (right panel) and the indicated concentrations of the sensor from which the reaction was measured over time in the steady phase of the reaction; (B) A classical Michaelis-Menten plot for the steady state phase of the reaction performed with the sensor in the absence (DMSO) or presence of the drug (compound **I**, 80 μM). The rates of the steady state phase are plotted as a function of sensor concentrations. Standard errors of

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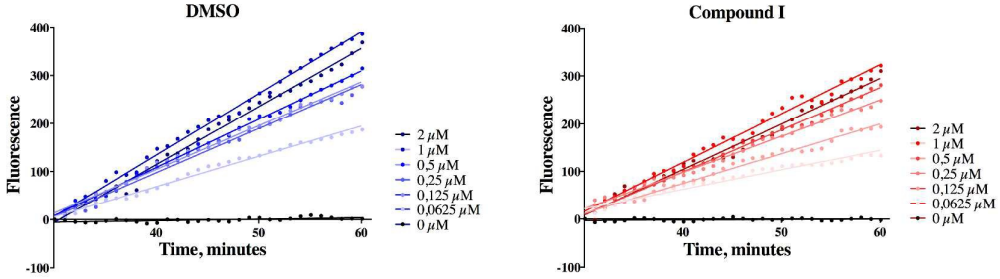
the mean of 10 or 13 independent experiments for samples with DMSO and compound **I**, respectively, are indicated.

A Linear least square fits from signals obtained in the steady phase of the reactions



B Michaelis-Menten plot (steady phase)

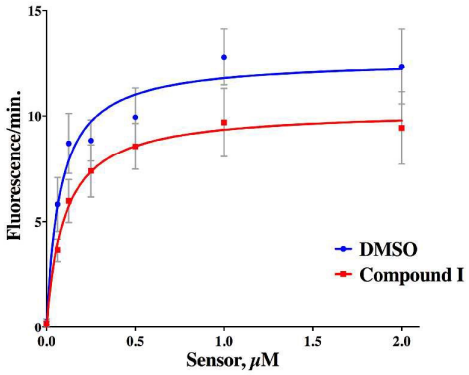


Fig. 4

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Fig. 5 HTopI mediated cleavage on a 5'-radiolabelled 67 bp DNA fragment in the presence of DMSO, 80 μM of CPT or 80 μM compound **I** as indicated in top of the figure. C is a control without added hTopI. C1 and C2 indicate the result of accumulated cleavage complexes.

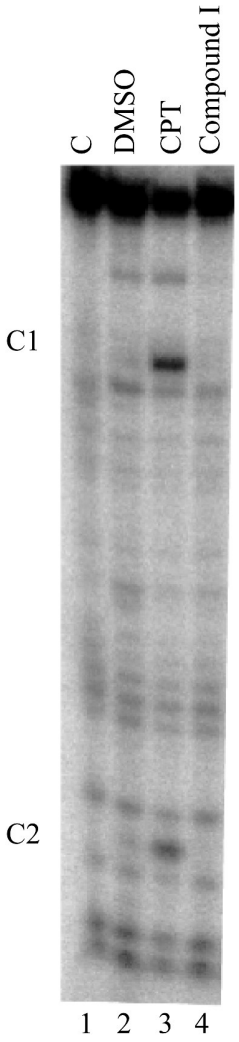

Fig. 5

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Fig. 6 (A) Right panel, schematic illustration of the anticipated reaction flow when hTopI was incubated with compound **I** and circular competitor DNA before addition of the 5'-phospho-sensor. If prevented in clamp-opening by compound **I** the enzyme may be unable to leave the circular DNA to react with the 5´-phospho sensor. In the absence of drug the enzyme clamp is anticipated to be able to open to release the competitor DNA. This will allow cleavage of the 5´-phospho-sensor. Left panel, is a graphical depiction of the result obtained when hTopI was pre-incubated with or without 80 μM compound **I** before molar excess of a circular plasmid DNA was added and the samples were incubated for additional 10 min after which the phosphorylated DNA sensor (1 μM final) was added and the reaction was followed over time; (B) Left panel, schematic illustration of the anticipated reaction flow when hTopI was incubated with compound **I** and linear competitor DNA before addition of the 5´-phospho-sensor. If the enzyme clamp is prevented in opening by e.g. compound **I** the enzyme may be able to dissociate from the competitor DNA by sliding along the DNA axis until it reaches the end. The enzyme may then be free to react with the sensor. Right panel, same as A (right panel) except that a molar excess of a linear DNA fragment was used as competitor instead of circular plasmid DNA; (C) Shows the reactions performed in the absence of drug in A and B for comparison. The circular plasmid DNA and the linear DNA fragment were present in a 1:1 base pair relation compared to the DNA sensor. HTopI is illustrated as a blue packman, the green arrow represent the fluorescence emitted when the quencher is removed.

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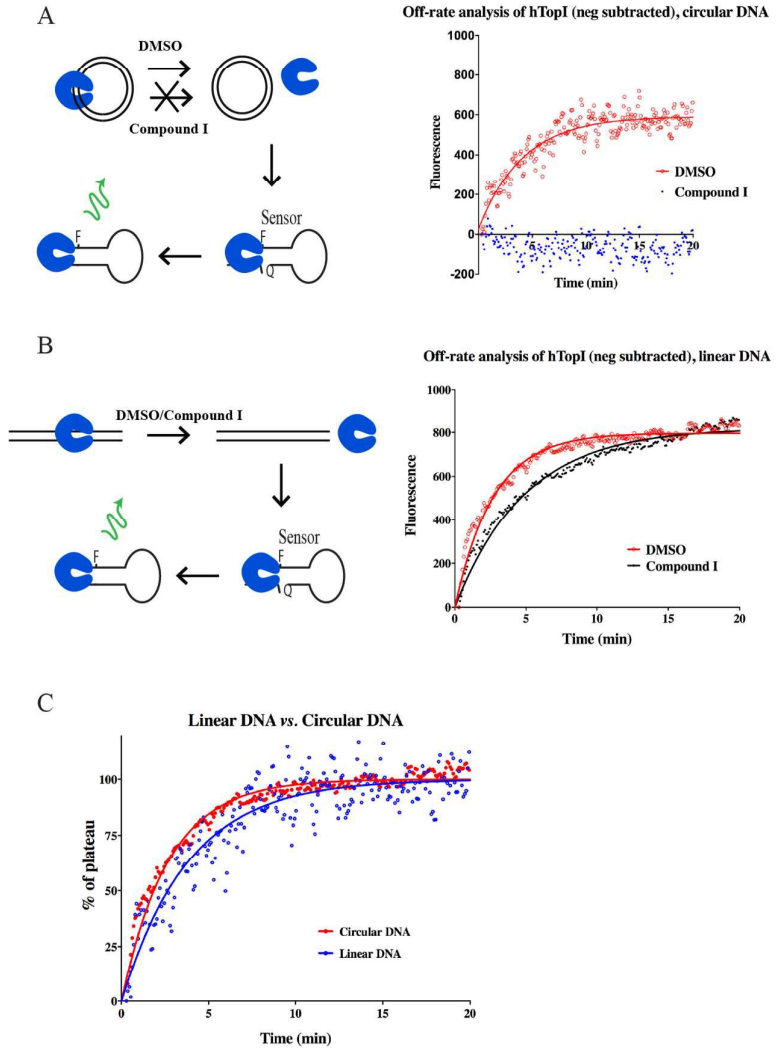

Fig. 6

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Table 1. The calculated reaction constants of hTopI catalysis in the presence of the drug solvent (DMSO) or 80 μM compound **I**.

Table 1, Michaelis-Menten (best fit values)
Kinetic parameters for hTopI on the DNA sensor +/- compound **I**

DMSO	
Vmax (Fluo./min.):	12.68 (±0.9734)
Km (μM):	0.07577 (±0.02747)
*Compound **I**, 80 μM*	
Vmax (Fluo./min.):	10.28 (±0.9476)
Km (μM):	0.09899 (±0.03905)