Supplementary information

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

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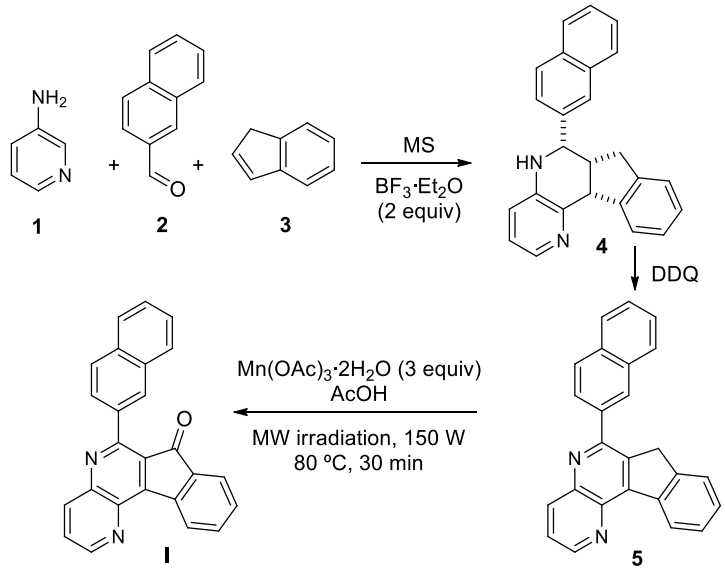
Fig. S1

(a) Scheme for the synthesis of 6-(2-naphthyl)-7*H*-indeno[2,1-c][1,5] naphthyridine-7-one **I**



Scheme 1.

(b) Experimental procedures

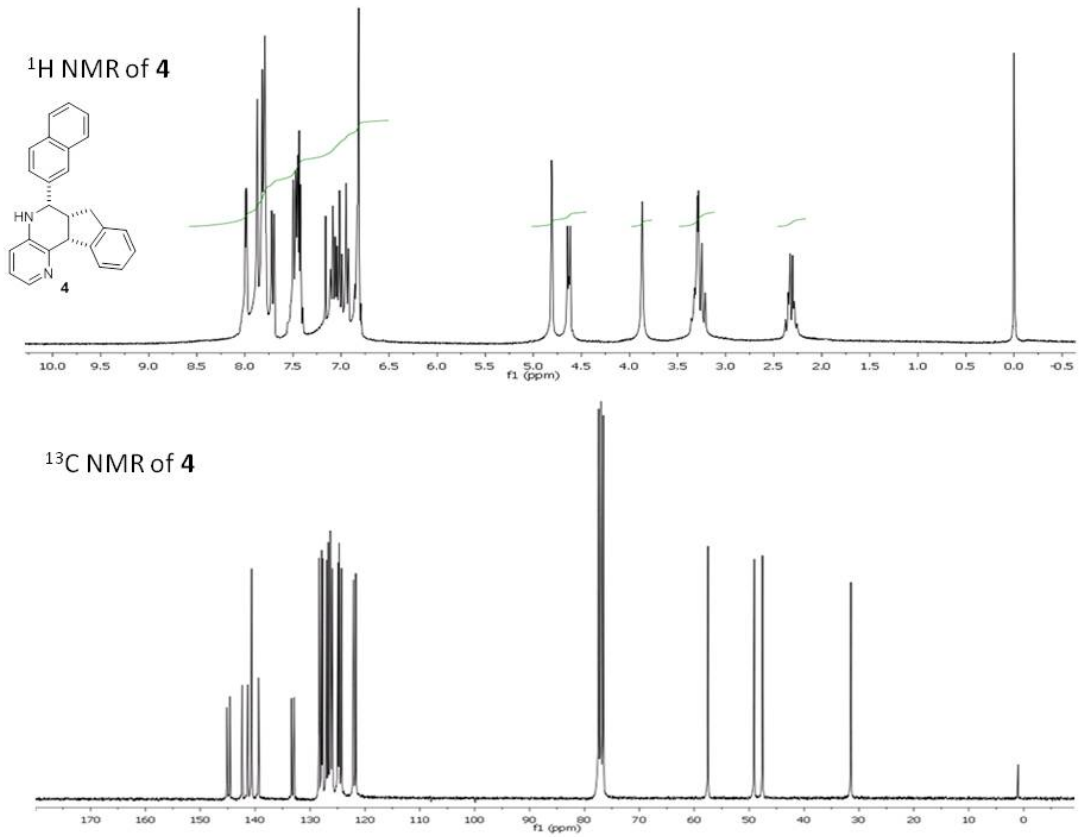
Preparation of 6-(2-naphthyl)tetrahydro-7*H*-indeno[2,1-*c*][1,5]naphthyridine (4). A mixture of 3-aminopyridine (10 mmol, 0.941 g), freshly distilled 2-naphthylaldehyde (10 mmol, 1.562g) in $CHCl_3$ (25 mL), indene (15 mmol, 1.735 ml) and two equivalents of BF_3•Et_2O (20 mmol, 2.461 ml) in the presence of molecular sieves (4Å) was stirred and heated at reflux for 36h. The molecular sieves were removed by filtration and the resulting solution was diluted with methylene chloride (50 ml), washed with a solution of NaOH 2M (50ml) and with water (50ml), extracted with methylene chloride (2x20ml), and dried ($MgSO_4$). Removal of solvent under vacuum led to an oil that was purified by flash column chromatography on silica gel using a gradient elution of 10 to 40% ethyl acetate in hexane to afford a mixture of compounds **4/5** (1.742 g, 50% and 0.344g, 10%, respectively) as yellowish solids after purification by flash chromatography. See data for compound **5** below. Data for compound **4**: mp 115-117 °C (ethyl acetate/hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.31 (dd, $^2J_{HH}$ = 14.3 Hz, $^3J_{HH}$ = 8.3 Hz, 1H, CH_2), 3.19-3.28 (m, 2H, HC_{6a}-CH_2), 3.85 (s, 1H, NH), 4.61-4.64 (m, 1H, HC_{11b}), 4.81 (s, 1H, HC_6), 6.76-6.83 (m, 2H, H_{arom}), 6.92-7.10 (m, 3H, H_{arom}), 7.41-7.49 (m, 3H, H_{arom}), 7.70 (d, $^3J_{HH}$ = 7.4 Hz, 1H, H_{arom}), 7.77-7.81 (m, 3H, H_{arom}), 7.87 (s, 1H), 7.98 (dd, $^4J_{HH}$ = 2.3 Hz, $^3J_{HH}$ = 4.1 Hz, 1H, H_{arom}) ppm; ^{13}C RMN (75 MHz, CDCl3) δ : 31.5 (CH_2), 47.6 (HC), 49.1 (HC), 57.5 (HC), 121.6 (HC_{arom}), 122.1 (HC_{arom}), 124.3 (HC_{arom}), 124.7 (HC_{arom}), 124.9 (HC_{arom}), 125.9 (HC_{arom}), 126.3 (2 HC_{arom}), 126.7 (HC_{arom}), 127.0 (HC_{arom}), 127.7 (HC_{arom}), 127.9 (HC_{arom}), 128.3 (HC_{arom}), 132.9 (C_{arom}), 133.4 (C_{arom}), 139.3 (C_{arom}), 140.6 (HC_{arom}), 141.3 (C_{arom}), 142.4 (C_{arom}), 144.6 (C_{arom}), 145.1 (C_{arom}) ppm; HRMS (EI) for $C_{25}H_{20}N_2$ $[M]^+$ calcd 348.1626 found 348.1631.

Preparation of 6-(naphthalen-2-yl)-7*H*-indeno[2,1-c][1,5]naphthyridine (5). To a solution of 6-(Naphthalen-2-yl)tetrahydro-5*H*-indeno[2,1-*c*][1,5]-naphthyridine **4** (1 mmol) in toluene (20 mL) was added DDQ (1 mmol, 0.227 g) and the mixture was irradiated with microwave at 150 W at 25 °C for 2 h. The formed solid was filtered off, the solvent of the resulting solution removed under vacuum leading to an oil that was purified by column chromatography on alumina (ethyl acetate / hexane 1:20) to afford 0.206 g (60%) of **5** as a white solid, mp 158-159 °C (ethyl acetate/hexane). ^{1}H RMN (300 MHz, $CDCl_3$): δ: 4.04 (s, 2H, CH_2), 7.32-7.36 (m, 1H, H_{arom}), 7.44-7.49 (m, 3H, H_{arom}), 7.53 (dd, $^3J_{HH}$

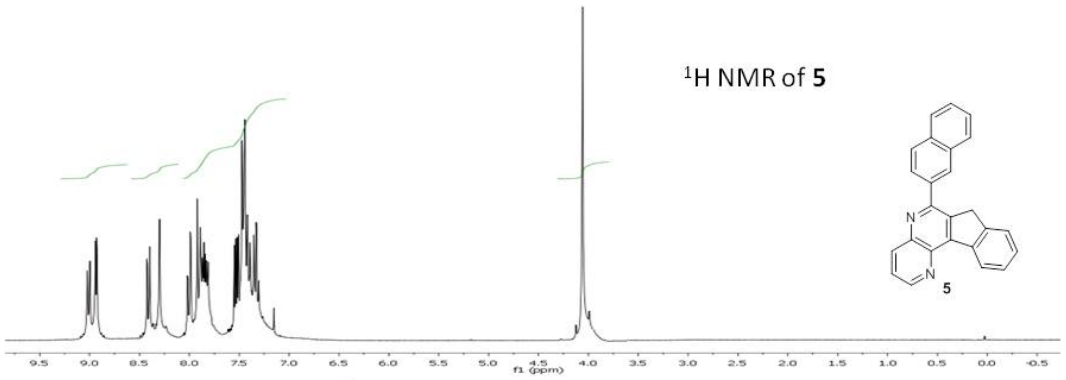
= 8.6 Hz, $^4J_{HH}$ = 4.1 Hz, 1H, H_{arom}), 7.81-7.96 (m, 4H, H_{arom}), 8.00 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 1.8 Hz, 1H, H_{arom}), 8.30 (s, 1H, H_{arom}), 8.41 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 1.8 Hz, 1H, H_{arom}), 8.93 (dd, $^3J_{HH}$ = 4.1 Hz, $^4J_{HH}$ = 1.8 Hz, 1H, H_{arom}), 9.01 (d, $^3J_{HH}$ = 7.5 Hz, 1H, H_{arom}) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ: 38.3 (CH_2), 124.1 (HC_{arom}), 124.6 (HC_{arom}), 126.6 (2C, HC_{arom}), 127.0 (HC_{arom}), 127.1 (HC_{arom}), 127.6 (HC_{arom}), 128.0 (HC_{arom}), 128.6 (HC_{arom}), 128.7 (HC_{arom}), 128.8 (HC_{arom}), 128.9 (HC_{arom}), 133.4 (C_{arom}), 133.7 (C_{arom}), 137.4 (C_{arom}), 137.5 (HC_{arom}), 137.6 (C_{arom}), 140.1 (C_{arom}), 140.6 (C_{arom}), 143.5 (C_{arom}), 144.6 (C_{arom}), 146.9 (C_{arom}), 150.4 (HC_{arom}), 156.6 (C_{arom}) ppm; HRMS (EI) for $C_{25}H_{16}N_2$ [M]+ calcd 344.1313 found 344.1318.

Preparation of 6-(naphthalen-2-yl)-7*H*-indeno[2,1-c][1,5]naphthyridine-7-one (I). To a solution of 7*H*-indeno[2,1-c][1,5]-naphthyridine **5** (1 mmol, 0.344 g) in acetic acid (10 mL) $Mn(AcO)_3$ (3 mmol, 1.6 g) was added. The mixture was under microwave irradiation at 150 W and 80 °C during 30 min. The formed manganese diacetate was filtered off, the reaction mixture was poured into water, neutralized by $NaHCO_3$, extracted with methylene chloride (20 ml) and dried over anhydrous sodium sulphate. The solvent of the resulting solutions was removed under vacuum leading to an oil that was purified by column chromatography on alumina (ethyl acetate / hexane 1:10) to afford 0.178 g of compound **I** as a yellow solid (50%), mp 215-216 °C (ethyl acetate/hexane). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.30-7.39 (m, 3H, H_{arom}), 7.40-7.63 (m, 3H, H_{arom}), 7.80- 7.92 (m, 4H, H_{arom}), 8.35- 8.41 (m, 2H, H_{arom}), 8.80 (d, $^3J_{HH}$ = 7.2 Hz, 1H, H_{arom}), 9.01-9.03 (m, 1H, H_{arom}) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ: 124.5 (HC_{arom}), 124.9 (C_{arom}), 126.4 (HC_{arom}), 126.9 (C_{arom}), 127.1 (C_{arom}), 127.4 (HC_{arom}), 127.7 (HC_{arom}), 128.9 (HC_{arom}), 130.0 (HC_{arom}), 131.4 (HC_{arom}), 132.9 (HC_{arom}), 133.2 (C_{arom}), 133.3 (C_{arom}), 134.3 (HC_{arom}), 134.7 (HC_{arom}), 134.8 (HC_{arom}), 135.0 (HC_{arom}), 137.6 (HC_{arom}), 138.0 (HC_{arom}), 139.4 (C_{arom}), 141.4 (C_{arom}), 148.0 (C_{arom}), 152.2 (HC_{arom}), 153.7 (C_{arom}), 157.5 (C_{arom}), 192.6 (C=O) ppm. HRMS (EI) for $C_{25}H_{14}N_2O$ $[M]^+$ calcd 358.1106 found 358.1114.

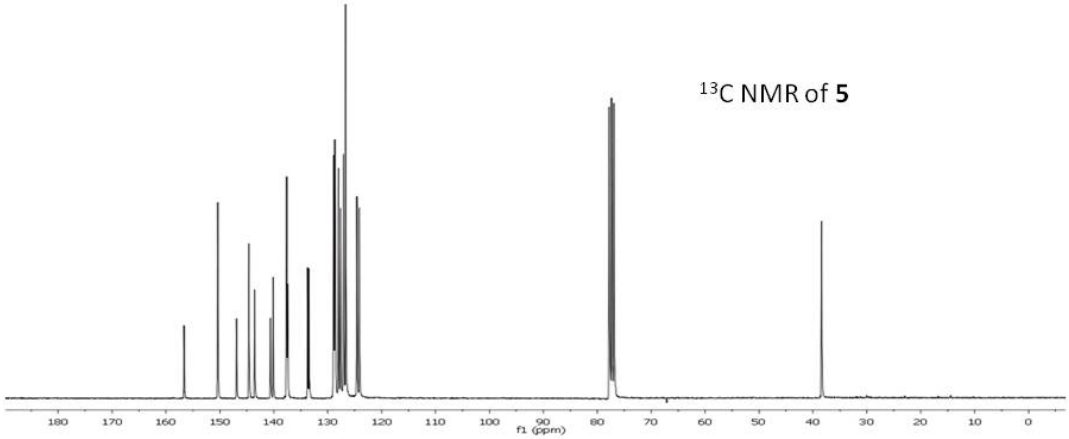
(c) ^{1}H and ^{13}C-NMR spectra of compounds 4, 5 and I



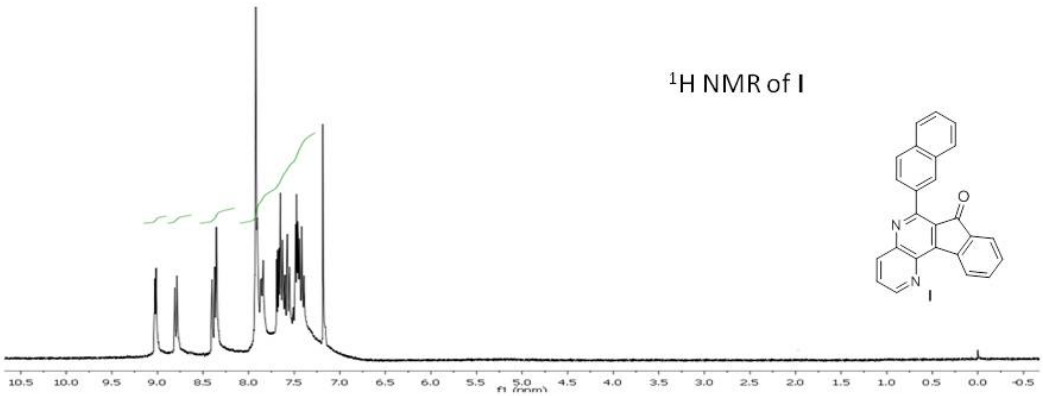
^{1}H NMR of **5**



^{13}C NMR of **5**



^{1}H NMR of **I**



^{13}C NMR of **I**

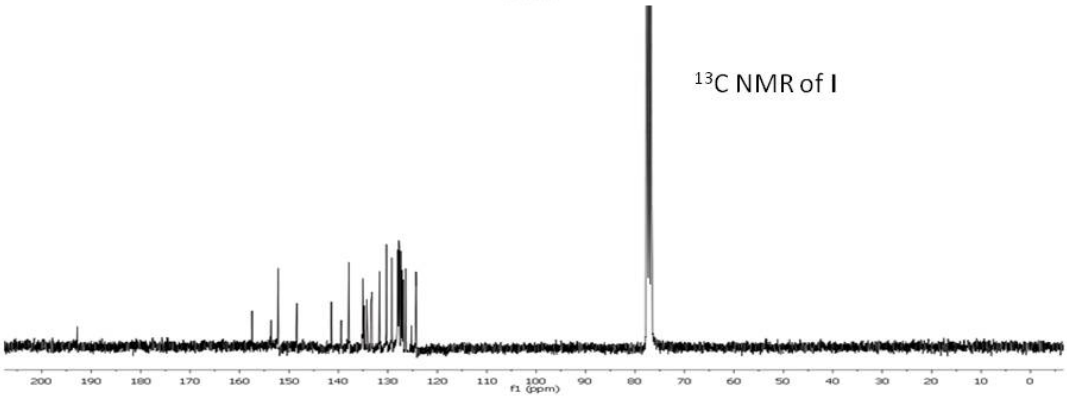


Fig. S2

Titration experiments were performed to investigate the effect of increasing concentrations of either compound **I** or CPT ranging from 10 to 120 μM on hTopI mediated relaxation. In the experimental setup 200 fmol of supercoiled plasmid DNA (pUC18) were mixed with the indicated drug concentrations or 2.5% (v/v) DMSO (the solvent of compound **I** or CPT) before purified hTopI was added and the reaction performed for 3 min. at 37 °C. The concentration of DMSO added to the drug-negative samples corresponded to the DMSO concentration in the drug containing samples. After incubation the reactions were terminated by the addition of 0.2% (w/v) SDS (final concentration) and proteinase K digested before analysis in a 1% agarose gel. The reaction products were visualized by EtBr staining and the results depicted above. Lane 1, control plasmid DNA without added hTopI, lane 2, positive control of relaxation where plasmid DNA was incubated with hTopI without drugs or DMSO, lane 3, same as lane 2 except for the addition of DMSO to the samples, lanes 4-8, the result of relaxation in the presence of indicated concentrations of CPT, lanes 9-13, the result of relaxation in the presence of indicated concentration of compound **I**. As evident from the figure the addition of compound **I** inhibited the conversion of supercoiled plasmid DNA to relaxed forms only at relative high concentrations (compare lanes 9-13 with lane 3), while CPT appeared a more effective inhibitor (compare lanes 4-8 with lane 3).

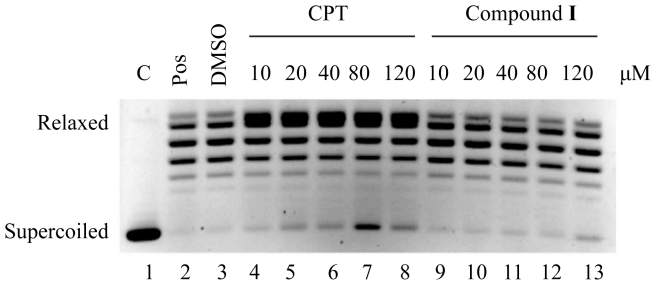


Fig. S3.

(A) Chemical structure of compound **II (Alonso et al, 2016)**; (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), 80 μM CPT (lane 4-6) or 80 μM compound **II** dissolved in DMSO (lanes 7-12). Lanes 7-9 show the result obtained when the DNA and drug were mixed immediately prior to addition of enzyme. Lane 10-12 is the same as lanes 7-9, except that the enzyme was pre-incubated with compound **II** for 10 min. at 37° C before addition of plasmid DNA. Reaction products were analyzed in a 1% agarose gel and visualized by subsequent EtBr staining.

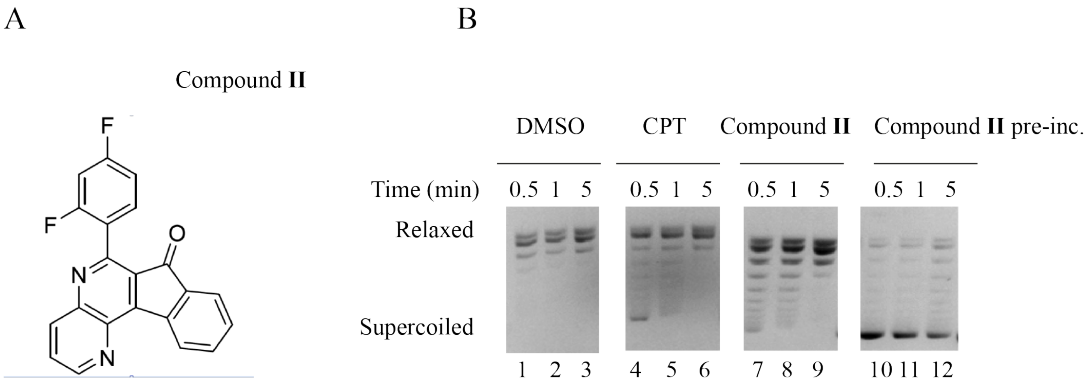


Fig. S4

Specificity of the sensor.

The hTopI sensor has been incubated with 200 fmol of hTopI (green) or T4 ligase (red) to test for specificity of the sensor. As negative controls similar experiments were performed using hTopI buffer (black) or T4 ligase buffer (grey) as substitutes for hTopI or T4 ligase. The mean of the data are plotted as function of the increase of fluorescence during time. SEMs are indicated by light color.

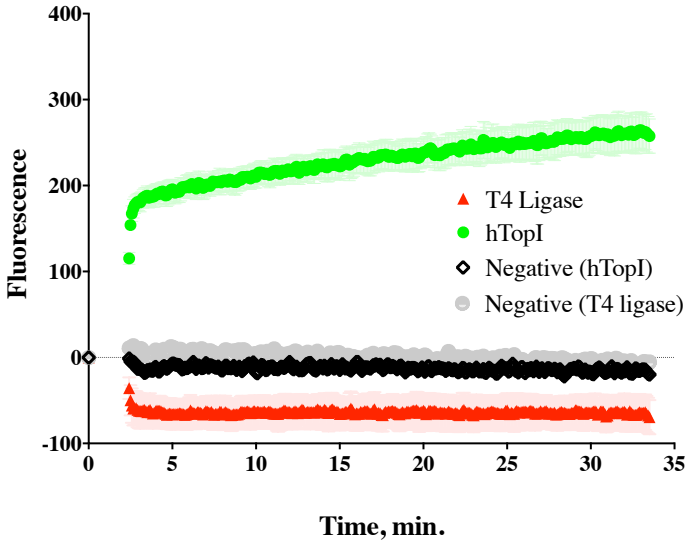


Fig. S5.
(A) Schematic representation of the burst profile with the relevant equations describing the reaction (modified from (Kristoffersen et al., 2015) and originally (Fersht, 1985)). When only analyzing the steady phase, the equations can be simplified compared to what was described earlier in (Kristoffersen et al., 2015). Here k_{cat} is the total turnover rate constant including substrate cleavage, relegation and dissociation. (The same rate constant was termed k_{steady} in (Kristoffersen et al., 2015)). See (Kristoffersen et al., 2015) for a more detailed description of the DNA sensor reaction including the initial burst phase.
(B) Schematic representation of the standard non-competitive inhibition (Strelow et al., 2012). E denoted the enzyme, I denote the inhibitor, S denotes the substrate and P denotes the product of the enzymatic reaction. The dot denotes that two or more reagents form complex. k denotes rate constants of association or dissociation. k_{cat} is the rate constant of the sum of the reactions needed for enzymatic turnover. The non-competitive inhibitor can bind the enzyme alone or in complex with the substrate. However, when present the drug prevent full enzymatic turnover (Strelow et al., 2012). This leads to lowering of the total reaction velocity seen by a reduced apparent maximal velocity (Vmax) due to lowering of the effective enzyme concentration. The apparent Km is unchanged if the rate constants of inhibitor association (k_2) and dissociation (k_{-2}) are similar for E to E•I and E•S to E•I•S as denoted in the scheme. In the case for hTopI and compound **I** this is observed only on short linear substrates as are explained in the text.

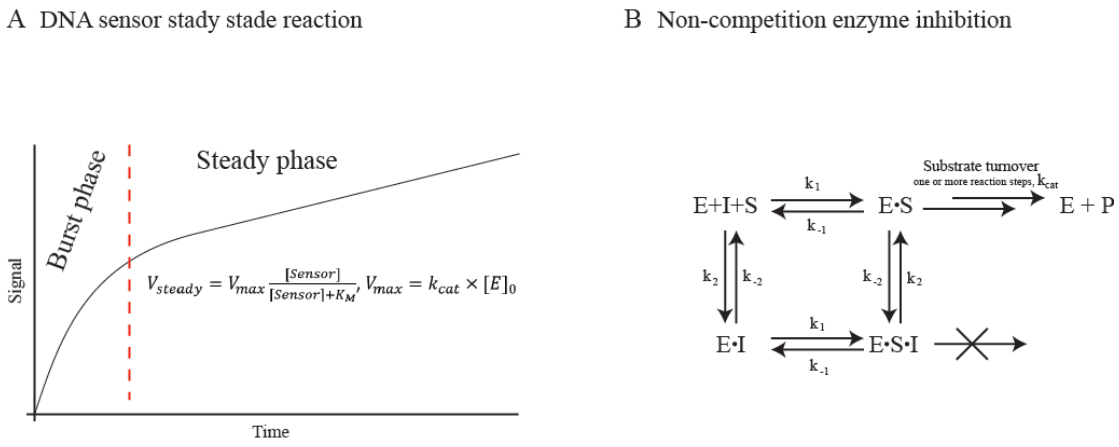
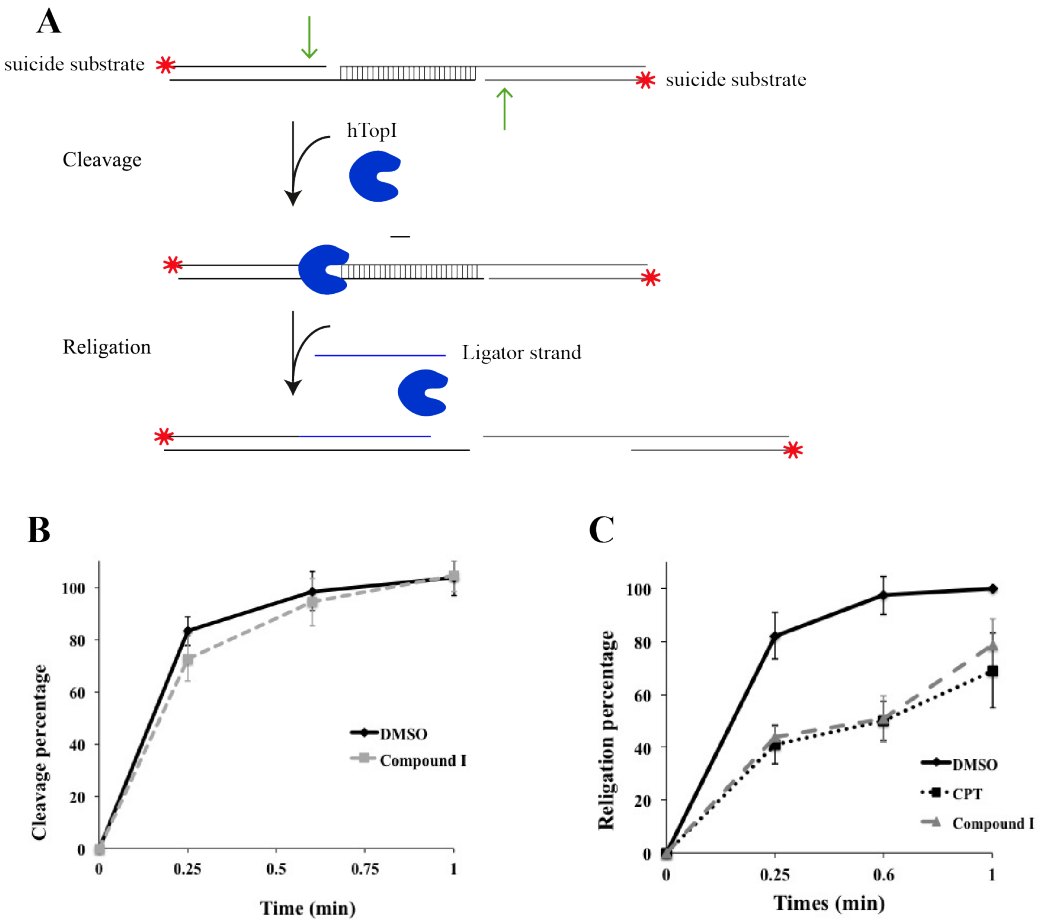


Fig. S6.
(A) Schematic depiction of the suicide substrate system. The cleavage substrate is composed of a 5'-radiolabelled (red asterisk) scissile strand (cleavage site indicated by green arrow) hybridized to a non-cleaved strand with a 5'-overhang having a palindromic sequence. This allows two substrates to dimerize to form double stranded DNA on both sides of the cleavage site, which is a prerequisite for hTopI mediated cleavage. Note, that ligation of the non-cleaved strand is prevented by 5'-phosphorylation. Addition of hTopI (blue packman) leads to cleavage and formation of cleavage complexes but ligation is prevented due to dissociation of a short oligonucleotide with the 5'-OH generated during ligation. Ligation to an external DNA strand can be initiated by adding molar excess of such a ligator strand with a 5'-OH end and having a sequence matching the non-cleaved strand. (B) and (C) show the result of analyzing the effect of compound I on the separate hTopI cleavage and ligation activities, respectively, using the suicide substrate system. Cleavage and ligation experiments were performed in parallel. Hence, the lack of effect of compound I on cleavage cannot be ascribed to an inactive drug since ligation was affected by compound I. As a positive control the effect of CPT, a known inhibitor of ligation, was included in the experiment assaying ligation. CPT has no effect on cleavage compound I was compared with DMSO alone. (B) Quantification of results obtained from hTopI DNA cleavage analyzed in the presence or absence of 80 μM Compound **I** for increasing time periods using the suicide substrate system illustrated in A. (C) Quantification of results obtained from hTopI DNA relegation analyzed in the presence of DMSO, 80 μM CPT or 80 μM Compound **I** for increasing time periods using the suicide substrate system illustrated in A.



Supplementary information material and method

Test of sensor specificity

The hTopI DNA sensor was prepared has reported in the manuscript material and method.
Activity measurements were carried out incubating 0.125 μM of the sensor with hTopI or T4 ligase (New England biolabs) in the presence of hTopI reaction buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 24 mM NaCl) or T4 ligase reaction buffer in a final volume of 25 μL. The components were mixed in two separate tubes, one containing the substrate and the second containing the hTopI or T4 ligase. Development of fluorescence was followed in a Mx3000P qPCR machine (Agilent Technologies, Inc.) and data were collected every 5 seconds for the first 30 minutes. Raw data were plotted as increase of fluorescence as function of time.

HTopI mediated cleavage

Oligonucleotides sequences for construction of suicide cleavage substrate (Fig S6 A) are as follows: AS88: 5'-
GCCTGCAGGTCGACTCTAGAGGATCTAAAAGACTTAGA-3'
AS90: 5'AAAAATTTTTCTAAGTCTTTTAGATCCTCTAGAGTCGACCTGCAGGC-3'
The oligonucleotide representing the cleavage strand (AS88) that contains a hTopI high affinity cleavage site (Fig. S6. A, green arrow) was 5'-radiolabelled prior to hybridization to the non-cleavage strand (AS90) by employing the T4 polynucleotide kinase reaction using [γ-^{32}P]ATP as phosphoryl donor. Unreacted [γ-^{32}P]ATP was removed by spin dialysis on a G-50 column (GE-healthcare). For hybridization, 10 pmol of radiolabelled AS88 oligonucleotide was mixed with 20 pmol of AS90 in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, heated to 95 °C for 5 minutes, and cooled slowly to room temperature. In order to prevent unspecific ligation of the 5'-OH of the non-cleavable strand, AS90 was phosphorylated at the 5'-OH prior to the annealing.
Prior to the cleavage reaction, 990 fmol hTopI was pre-incubated with 80 μM CPT or 80 μM Compound **I** for 10 minutes at 37 °C 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. Control samples with no addition of drug were supplied with an equal volume of DMSO (3 % (v/v) final concentration).
Cleavage reactions were initiated by addition of 20 nM radiolabelled suicide DNA substrate AS88/AS90 in a final reaction volume of 30 μl. 5 μl aliquots of the reaction mixture were terminated after given periods of time by the addition of 0.7 % (w/v) SDS. DNA was 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 radiolabelled DNA bands were visualized by PhosphorImager (Bio-Rad). The extent of cleavage in each sample was quantified using the QuantityOne software from Bio-Rad and normalized to 100 % cleavage for the reaction performed in presence of DMSO after 1 minute of incubation. The results are plotted as the average of three independent experiments and error bars indicate standard deviations.

HTopI mediated ligation

Active hTopI-DNA cleavage complexes were generated by incubating 20 nM of suicide DNA substrate AS88/AS90 with 275 fmol hTopI at room temperature for 30 minutes following 15 minutes incubation at 37 °C 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. Prior to addition of the ligator oligonucleoutide, the TopI-DNA cleavage complexes were pre-incubated with 80 μM CPT or 80 μM Compound **I** for 10 minutes. Control samples with no addition of drug were supplied with an equal volume of DMSO.

The ligation reaction was initiated by addition of 2 μM ligator oligonucleotide (AS915'-AGAAAAATTTTT) to the reaction mixture. 5 μL aliquots of the reaction was terminated after given periods of time by the addition of 0.7% (w/v) SDS (final concentration). Samples were precipitated, trypsin digested analyzed and quantified as described for hTopI mediated cleavages.

Supplementary information references

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