A Novel Homogenous Assay for Topoisomerase II Action and Inhibition

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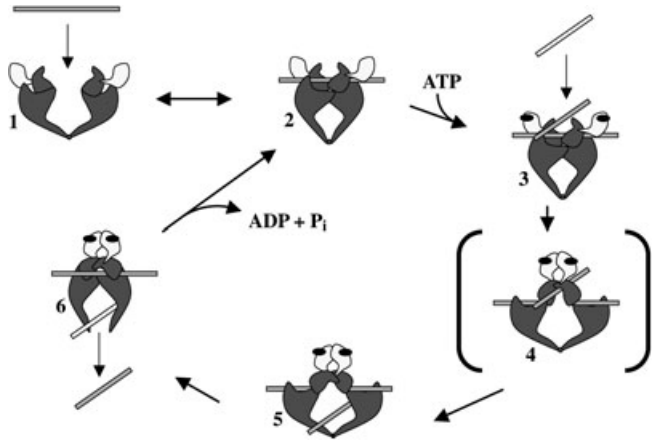
Topoisomerase II is the only enzyme able to cleave and religate double-stranded DNA; this makes it essential for many vital functions during normal cell growth. Increased expression of topoisomerase II is a common occurrence in neoplasia, and different topoisomerase II inhibitors have indeed been proven to be powerful anticancer drugs. For this reason, the topoisomerase II catalytic cycle has attracted strong interest, but only a few techniques contributing to studies in this field have emerged. All of the currently used conventional methods to elucidate the action and inhibition of topoisomerase II require separation steps and are therefore unsatisfactory in terms of sensitivity, speed, and throughput. Here, for the first time, we present an assay that works in homogenous solution. The assay is based on dual-color fluorescence cross-correlation spectroscopy (DC-FCCS) and allows monitoring of topoisomerase II action and, especially, detection and discrimination of different topoisomerase II inhibitor classes. The effectiveness of our new assay was confirmed by measuring the effects of a catalytic inhibitor (novobiocin) and a topoisomerase poison (m-AMSA) with bacteriophage T4 topoisomerase as a model system, thus showing the strategy to be easy, fast, and extremely sensitive. Further development of the DC-FCCS-based assay and subsequent application in high-throughput drug screening of new anticancer drugs is proposed and discussed.

Introduction

Eukaryotic type II DNA topoisomerases are ubiquitous enzymes with nuclear intracellular localization. They play important roles in many metabolic processes, including replication, transcription, recombination, and chromosome condensation/decondensation.[1,2] The cellular function of type II topoisomerase consists of the regulation of the topological state of DNA by introducing a transient double-strand break into one DNA before a second DNA molecule passes through the gate. In fact, topoisomerase II is the only kind of enzyme able to cleave and religate double-stranded DNA.[3] This is achieved through an ATP-dependent catalytic cycle. One round of this cycle starts with the binding of a first DNA molecule to topoisomerase II. This DNA will be cleaved to contain the gate and is therefore referred to as the *gate* DNA (G-DNA). Binding of the G-DNA induces a conformational change in topoisomerase II (**1** and **2** in Scheme 1). In an ATP-assisted process, a second DNA molecule (called *transported* or T-DNA) is bound and fixed (**3** in Scheme 1). In the next steps, the G-DNA is cleaved by the enzyme and the T-DNA is moved through the gate, before religation of the double-strand break. Cleaving and rejoining of the G-DNA is mediated by two tyrosine residues in the topoisomerase dimers through a covalently attached phospho-tyrosyl complex as intermediate state (**4** in Scheme 1). With release of the T-DNA and dissociation of ADP+P_i, one catalytic cycle is finished. In the case of the yeast type II DNA topoisomerase, the structural basis for the decatenation process was shown to be a heart-shaped dimeric protein with a large central hole that undergoes dramatic conformational changes upon ATP binding.[4,5]



Scheme 1. *The catalytic cycle of type II DNA-topoisomerase, as described in the text (taken from ref. [4], with permission).*

Several inhibitors for the different steps in the catalytic cycle have been described and characterized.[6–8] Inhibitors of the religation step promote accumulation of a covalently attached DNA–protein complex, the so-called cleavable complex. In this complex, DNA can be cleaved, but not religated. These inhibitors were found to induce cell death and are therefore named *topoisomerase poisons*. The detailed mechanisms of apoptosis induction by topoisomerase poisons are still unclear,[9] but it has been shown that DNA repair mechanisms are involved in processing the huge DNA damage resulting from the stabilized cleavable complexes.[10,11] A second kind of topoisomerase II

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inhibitor is represented by compounds that do not show stabilization and accumulation of the cleavable complex, but block the catalytic cycle at other steps. They are called *catalytic inhibitors*.[3]

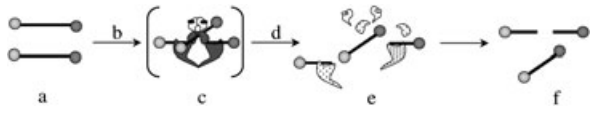
In contrast to normal, untransformed cells, many rapidly proliferating cancer cells show high expression levels of type II DNA topoisomerase, making this enzyme an ideal target for anticancer drugs.[9,12–14] In fact, topoisomerase II is the target for some of the most active antitumoral drugs currently used.[3,8] Among the topoisomerase II-targeted agents currently in clinical use are etoposide, teniposide, doxorubicin, mitoxantrone, and amsacrine,[15] all of them topoisomerase poisons. However, catalytic inhibitors also appear to have clinical potential.[3] Originally, topoisomerase II catalytic inhibitors were essentially defined by antibacterial coumarin-based drugs, such as novobiocin and coumermycin.[16] Currently, catalytic inhibitors that display high activity against eukaryotic topoisomerase II activity are typified by drugs such as merbarone, aclarubicin, staurosporine, and mitindomide, which reflect a variety of inhibitory mechanisms.[15] Two new therapeutic approaches based on the combination of a poison with a catalytic inhibitor in cancer treatment have been designed.[3]

Because of their evident pharmacological and clinical interest, a great effort is devoted to the discovery of new topoisomerase II inhibitors.[17,18] To achieve this goal, assays are needed that are fast and efficient whilst accomplishing both identification of inhibitory compounds and distinguishing between poisons and catalytic inhibitors. However, conventional methods to identify and characterize new topoisomerase II inhibitors are often unsatisfactory in terms of sensitivity, speed, and throughput—mainly because they require at least one separation step, such as electrophoresis. In this work we describe a new separation-free assay that operates in homogenous solution and is based on the concept of dual-color fluorescence cross-correlation spectroscopy (DC-FCCS) to detect compounds with different inhibitory effects on type II DNA topoisomerase and to analyze their effects. A short introduction to the single-molecule sensitive DC-FCCS technique can be found in the Experimental Section, whilst a more detailed theoretical and experimental background, as well as biological and biochemical applications of DC-FCCS, is described elsewhere.[19–23] Our results demonstrate that this new assay is useful for the study and discrimination of both kinds of topoisomerase II inhibitors and can potentially be further developed for application in high-throughput screening of new anticancer drugs.

Results and Discussion

Theoretical background of the new assay

As the G-DNA trapped in the intermediate complexes of the topoisomerase II catalytic cycle is in a cleaved state,[3,8] DC-FCCS can be used to detect the double-strand break, provided that the DNA substrate carries two spectrally different fluorophores at the opposite 5′-ends. Therefore, the principle of the assay developed in this work is as follows: linear, double-fluorophore-labeled (Rhodamine green and Cy5) double-stranded DNA serves as a substrate for the topoisomerase II catalytic cycle analyzed by DC-FCCS. The overall fraction of the intermediate state with the cleaved G-DNA covalently linked to topoisomerase II (4 and 5 in Scheme 1) should presumably be constant and small. The main criterion to distinguish both types of topoisomerase II inhibitors is the sign of the change in the concentration of intermediate complexes. Addition of a topoisomerase poison would result in an increased amount of double-strand breaks, whereas a catalytic inhibitor would prevent cleavage. Liberation of the cleaved G-DNA from the complexes into singly labeled fragments is necessary for detection of the cleavage when using DC-FCCS, because independent diffusing molecules are required to obtain decreased cross-correlation amplitudes. The degradation of the protein component of the complexes can easily be achieved by digestion with proteinase K. The overall concept of the assay is depicted in Scheme 2.



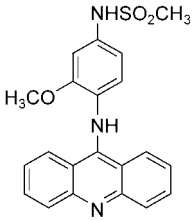
Scheme 2. *Concept of the new assay. a) Rhodamine green and Cy5 doubly labeled DNA serves as substrate. The bar between the 5′-attached labels represents double-stranded DNA. b) Addition of topoisomerase II, ATP, and a topoisomerase poison. c) The accumulated intermediate complex with the G-DNA cleaved and covalently attached to the enzyme. d, e) Proteinase K is used to degrade the protein component of the complex. f) Intact T-DNA and cleaved G-DNA fragments are freely diffusing after digestion.*

Carrying out DC-FCCS measurements in the absence of inhibitor, one would expect a small decrease in the cross-correlation amplitude after proteinase K digestion. In the presence of perfect topoisomerase poisons, the expected maximal decrease of the cross-correlation amplitude would be 50% of initial value, since only the G-DNA (but not the T-DNA) would be cleaved. An ideal catalytic inhibitor would result in stable cross-correlation amplitudes (in relation to the initial values) after proteinase K digestion.

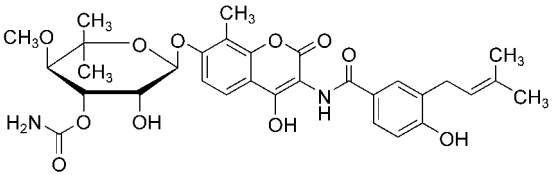
The model system used

In our experiments we used T4 bacteriophage type II DNA topoisomerase, which serves as a model protein for mammalian type II topoisomerases.[25] In fact, T4 topoisomerase II has previously been shown to exhibit striking similarities to eukaryotic topoisomerase II.[24–27]

To verify the ability of our assay to distinguish between topoisomerase poisons and catalytic inhibitors, we used the known and commercially available compounds *m*-AMSA and novobiocin, respectively. *m*-AMSA is the commercial hydrochloride salt of amsacrine, a topoisomerase poison currently applied in the clinical treatment of acute myeloid leukemia.[3] Novobiocin is a coumarin-based drug that blocks the DNA strand passage activity of the prokaryotic type II enzyme, DNA gyrase,



m-AMSA



novobiocin

by interfering with the ability of the enzyme to bind its ATP cofactor.[15]

The double-fluorophore-labeled 38-mer dsDNA substrate contained Rhodamine green and Cy5, and was assembled from oligonucleotides containing a very strong *m*-AMSA-inducible cleavage site for the T4 topoisomerase. This sequence has been described previously[24] and had been designed on the basis of a previous mutational analysis of a moderately strong cleavage site.[28]

DC-FCCS-based analysis of the effects of both kinds of inhibitors

Our results show that the new described assay does in fact allow efficient analysis and quantification of the percentage of dsDNA substrate molecules present in the cleavable complexes of the topoisomerase II catalytic cycle under the experimental conditions used, both in the absence and in the presence of either a topoisomerase poison or a catalytic inhibitor.

After incubation of doubly labeled dsDNA with T4 topoisomerase II in the absence of inhibitor and digestion with proteinase K, the normalized cross-correlation amplitude decreased by 7.5%, relative to the values obtained immediately after mixing (Figure 1a). By taking account of the corresponding autocorrelation amplitudes and applying Equation (4) (see Experimental Section), it was possible to estimate an average

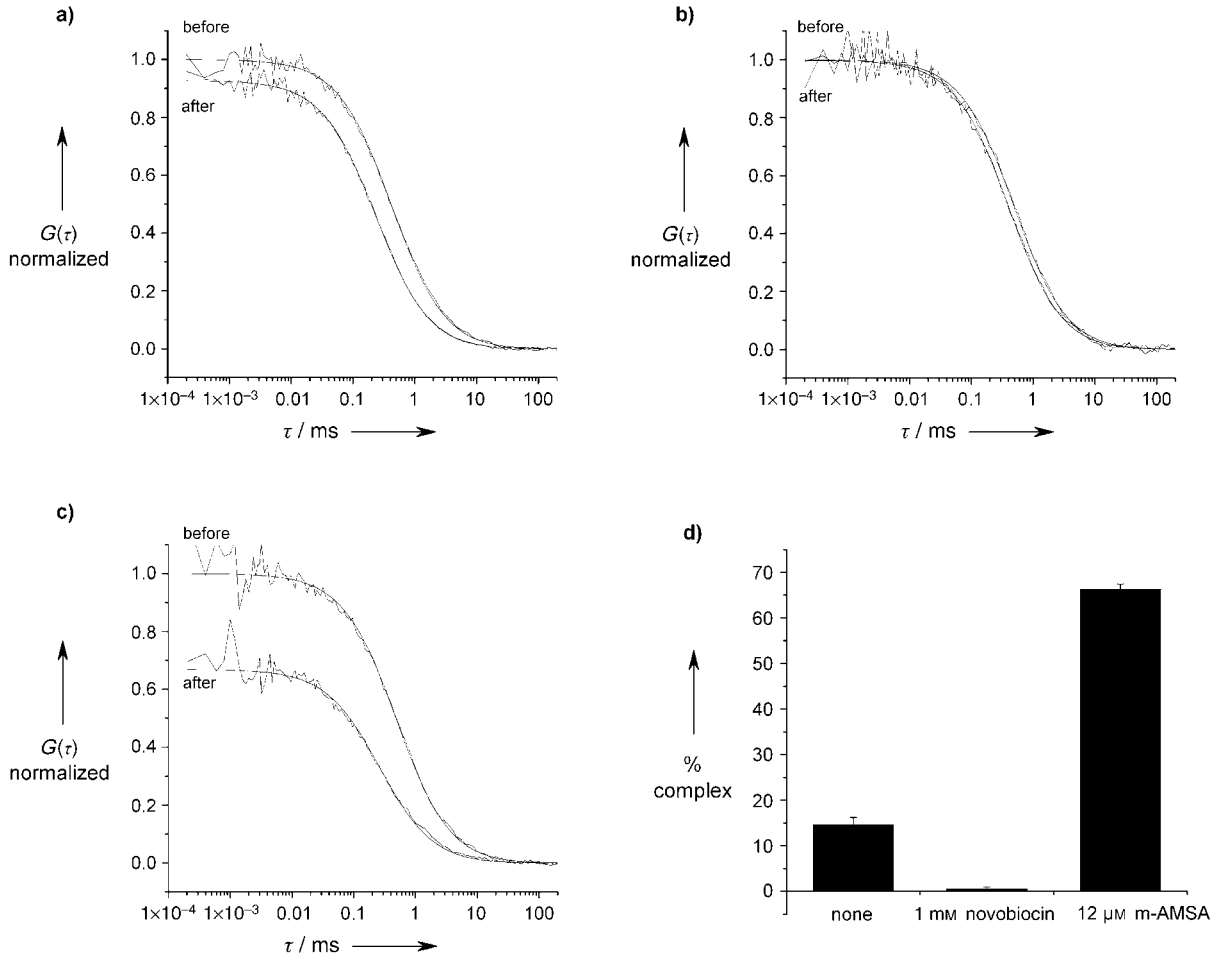


Figure 1. *a–c) Normalized cross-correlation and fit curves before and after proteinase K digestion. Curves are averaged from at least two independent measurements a) in the absence of inhibitor, or in the presence of b) 1 mM novobiocin and c) 12 μM m-AMSA. d) Calculated percentages of intermediate complexes from measurements shown in a–c.*

fraction of approximately 15% of the cleavable complex (Figure 1d). The normalized curves as plotted in Figure 1 are to be read as follows. The important criterion used to estimate relative changes in the amount of doubly labeled molecules (as an indicator of DNA cleavage) is the amplitude $G(0)$. With normalization, the initial amplitude obtained from the reaction direct after mixing is set to 1. Any topoisomerase-mediated cleavage of DNA will deplete the amount of doubly labeled substrate and therefore lead to a reduced amplitude, that is to say that the *y*-axis is a direct and sensitive indicator of the relative amounts of molecules carrying *both* labels.

Experiments in the presence of the catalytic inhibitor novobiocin showed almost no decrease in cross-correlation after digestion with proteinase K (Figure 1b). The fraction of cleavable complex observed without inhibitor was reduced to near zero after inhibition with novobiocin (Figure 1d), as anticipated above in our discussion of the theoretical background of the assay. The same experiment carried out in the presence of the known topoisomerase poison *m*-AMSA (Figure 1c) gave rise to a very remarkable decrease in cross-correlation after incubation and digestion with proteinase K. In fact, the cross-correlation amplitude decreased by almost 40%, a value very close to that theoretically predicted for perfect topoisomerase poisons. Furthermore, as expected, the amount of G-DNA trapped in cleavable complexes increased dramatically. By using Equation (4), we were able to estimate that up to 66% of the G-DNA was trapped in cleavable complexes after incubation with *m*-AMSA (Figure 1d). This maximum observed value of cleavage is in accordance with data obtained by others using more conventional assays.[24] As any dsDNA-cleaving agent (e.g., nuclease contamination) would lead to reduction in the relative cross-correlation amplitude, it is important to conduct negative controls in the absence of the topoisomerase II enzyme. We performed these control experiments and obtained stable relative cross-correlation amplitudes, confirming that no nuclease contamination was present in buffers or proteinase K batches (data not shown). The T4 topoisomerase II preparation itself was also free from nuclease contamination, as cross-correlation amplitudes were stable unless proteinase K was added. This confirms that the measured effect is due solely to the topoisomerase reaction itself.

The data summarized in Figure 1 confirm that the different behavior of topoisomerase II inhibitors *m*-AMSA and novobiocin could clearly be discriminated by the new assay used in our experiments.

Figure 2 shows the dependence of the percentage of accumulated cleavable complexes on the concentration of inhibitor. As expected, both inhibitors showed completely different effects. For *m*-AMSA, the amount of intermediate states increased up to 66% over a narrow concentration range of about one order of magnitude (0.5–12 μM), whereas in the case of novobiocin a concentration range of four orders of magnitudes (0.1–1000 μM) was necessary to decrease the percentage of complexes from the initial 15% value to zero (Figure 2). Higher concentrations of *m*-AMSA were tried, but led to massive formation of aggregates and therefore made DC-FCCS measurements impossible (results not shown).

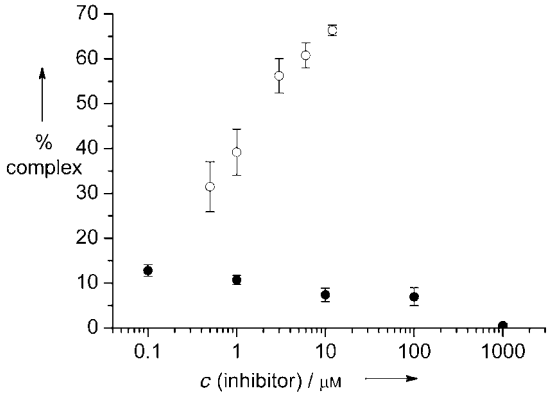


Figure 2. *Concentration dependence of the percentage of cleavable complex accumulated in the presence of the two topoisomerase II inhibitors. Data points represent averages from at least two independent measurements. ○: 0.5, 1, 3, 6, and 12 μM m-AMSA were used; ●: 0.1, 1, 10, 100, and 1000 μM novobiocin were tested.*

Effects of both kinds of inhibitors on diffusion time of DNA substrate

As an additional parameter to analyze binding of topoisomerase II to its DNA substrate, the mobility of labeled DNA molecules was evaluated. Diffusion times obtained from the single-component fit of autocorrelation curves are used for this purpose. The diffusion time τ_d of the molecules is proportional to the cubic root of the molecular weight ($\tau_d \propto \sqrt[3]{M}$). DNA bound to the dimeric topoisomerase II (M_r of the complex: 304 kDa) should have a diffusion time 2.3 times higher than that of free DNA (25 kDa).

Table 1 summarizes the results obtained in relation to the effects of both kinds of inhibitors on the diffusion times of DNA substrate derived from the fitting procedure of autocorrelation curves in both the green and the red channel. In all cases, average diffusion times after incubation and proteolytic digestion of topoisomerase were lower than those estimated from the autocorrelation curves obtained immediately after mixing. In experiments in the absence of inhibitor and in the presence of *m*-AMSA, the ratio of τ_d values obtained before and after in-

Table 1. *Effect of topoisomerase II on the DNA substrate diffusion time under different inhibitory conditions.*

Treatment	τ_d [ms] green channel			τ_d [ms] red channel		
	Before (B)	After (A)	Ratio B/A	Before (B)	After (A)	Ratio B/A
No inhibitor	0.176	0.106	1.66	0.434	0.242	1.79
12 μM *m*-AMSA	0.177	0.104	1.70	0.447	0.253	1.77
1 mM novobiocin	0.128	0.111	1.15	0.359	0.284	1.26

Diffusion times of the labeled DNA are derived from the fitting procedures of the autocorrelation curves, before and after incubation and digestion with proteinase K. Note that in general the diffusion times of the red labeled particles are higher due to the larger volume element produced by diffraction of light with longer wavelength.

cubation and proteolysis was 1.7–1.8. These data indicate that in the catalytic cycle the overall saturation of the substrate with topoisomerase II is the same in the absence of inhibitor and in the presence of *m*-AMSA. In contrast, experiments with novobiocin produced only a small change in the diffusion time, by a factor of about 1.2 (Table 1). This finding confirms that novobiocin interferes with DNA binding (steps 1–3 in Scheme 1).

Effects of sequential incubation with both inhibitors

When mixtures of both inhibitors were used, the result was determined by the order of addition. Addition of 1 mM novobiocin and then (after a first incubation) of 12 μM *m*-AMSA gave the same result as the experiments with 1 mM novobiocin alone, while addition of both inhibitors in the reverse order gave exactly the same result as obtained for *m*-AMSA only (results not shown). In conclusion, the effect was always determined by the first added compound. This finding can be explained by the fact that the topoisomerase II reaction consists of a catalytic cycle that proceeds in one direction only.[1–3]

Comparison between DC-FCCS and other conventional techniques

Inhibition of topoisomerase II activity is usually measured in a DNA decatenation assay with either agarose gel electrophoresis or a centrifugation step to separate the decatenated DNA.[15,29] In the gel assay, DNA is observed after electrophoresis by staining with ethidium bromide and observation in a transilluminator under UV light. The currently used centrifugation assay requires the use of radiolabeled DNA from trypanosomes grown in the presence of tritium-labeled thymidine. These assays are cumbersome and time-consuming, have relatively low sensitivity, and are difficult to apply to high-throughput screening. The new measurement strategy chosen here makes use of DC-FCCS and therefore has several advantages over traditional biochemical analysis methods. First of all, it works completely in solution, which eliminates the need for time-consuming separation steps such as electrophoresis and chromatography. Another advantage of the technique is its single-molecule sensitivity,[19–22] which allows the use of low concentrations and small volumes in order to minimize sample consumption. To compare the requirements of enzyme amount and purity for the conventional assay and our new assay, it can be stated that nuclease contamination has to be strictly avoided in both approaches, as this would lead to wrong results. Assays for distinguishing inhibitor types, like the one we are dealing with here, in general need larger amounts of topoisomerase II (excess over the substrate) than simple catalytic inhibition assays in which the mechanism of action of the inhibitor cannot be judged. Therefore, the only way to reduce the required amount of enzyme is to lower substrate concentrations and/or reaction volumes. The DC-FCCS detection technique uses an effective measurement volume of a few femtoliters and allows for a drastic reduction in reaction volume. In addition, it might be possible to establish working protocols using lower topoisomerase II and substrate concentrations. This is mainly dependent on the behavior of the enzyme in terms of binding properties. The sensitive optical detection would allow for further reduction in substrate concentration of about two orders of magnitude compared to the amount used here.

Although no example has been published yet, other existing optical techniques might also work well for observing topoisomerase action and inhibition and subsequently be suitable for screening purposes. In particular, strategies employing fluorescence resonance energy transfer (FRET) would be imaginable for detection of topoisomerase-induced double-strand breaks in DNA. As with DC-FCCS, labeling with two compounds would be required for FRET measurements. Note, though, that for FRET a spectrally overlapping donor–acceptor pair is needed, while in contrast DC-FCCS ideally requires two spectrally distinct fluorophores. However, FRET has a major drawback: the most important difference between FRET and DC-FCCS techniques is the freedom in choice of dye positions. While FRET requires the donor and acceptor to be positioned (and moved during reaction) within the characteristic radius R_0 (usually in the 5 nm range), DC-FCCS detects doubly labeled species independently of inter-dye distance; this allows much larger separations. Especially in the present topoisomerase study, this circumstance dramatically simplifies the choice of noninfluencing fluorophore positions—outside the topoisomerase binding site, for example—therefore avoiding conflicts and artifacts.

Outlook: Possible application of the DC-FCCS topoisomerase II assay for high-throughput screening of new inhibitory compounds

The assay developed in this paper was invented and brought to work to suggest the usefulness of DC-FCCS for identification of new, valuable compounds for application in anticancer therapy. The next task would now be to adapt our assay and the technique for high-throughput screening and measurement of unknown inhibitors. To that end, one of the major problems that need to be overcome is the sequence or *hot spot* problem. Topoisomerase II does not have a specific recognition target as known for, for example, class II restriction endonucleases. Nevertheless, the enzyme acts preferably on some so-called *hot spot* sites within a DNA sequence, rather then in a randomly distributed fashion over the whole sequence. The same is true for inhibitory compounds and especially for topoisomerase poisons: their inhibitory effect may vary depending on the sequence on which the cleavable complex is trapped. These sequence effects are not so prominent with some catalytic inhibitors, especially those that block DNA binding.

The results described here were obtained with a fixed 38 bp DNA hot spot, which is a known strong *m*-AMSA-inducible cleavage site. At its current stage, the assay allows for fast and convenient screening of *unknown* inhibitors acting on a *known* site. It is also easily possible to establish an array of different DNA sites, and to use this to identify the *unknown* hot spot of a *known* compound. To estimate the size of such an array, one

can consider the historically used substrate: kinetoplast DNA (kDNA). The major minicircle of kDNA is 2515 bp in length; this would mean that, with an average substrate length of about 40 bp, one 96-well plate with all different hot-spot sites would be sufficient to cover the whole kDNA.

The ultimate goal in topoisomerase II inhibitor screening would of course be to identify *unknown* inhibitors with *unknown* hot spots. To this end, we propose to employ the DC-FCCS-based assay in combination with the screening of an array of different hot spots. Such a set of sequences might be obtained from known hot spots, including their mutational analysis, and should ideally represent an overall consensus for virtually any inhibitor. It should be noted that we also tried to increase the length of a single substrate, but obtained huge aggregates with DNA > 200 bp, which made DC-FCCS measurements impossible. This phenomenon is probably due to a kind of network formed by topoisomerase II molecules acting on different partner strands. However, by the array approach, screening should be accessible, although for each individual compound to be screened, one array (96-well plate) would be necessary. For synthesizing, annealing, and distribution of the substrates among micro- or nanocarriers, laboratory automation is mandatory. For that reason, we did not perform such experiments but encourage biotech companies to do so.

Conclusion

The assay described in this work shows the usefulness of the DC-FCCS detection technique for following biochemical reactions in homogenous solutions in vitro. When we applied it to the topoisomerase II system, we were able to distinguish different inhibitors without any of the separation steps required in all other previously known techniques. The single-molecule sensitive optical method we used here is ideally suited to meet the challenging measurement requirements for possible high-throughput analysis. Indeed, the technique itself has already been further developed to serve as a high-throughput screening method.[30] Tremendous reductions in measurement times, achieved by coincidence analysis,[31] together with the emergence of suitable carriers offer the potential for automation of the whole process. In summary, our assay represents a sensitive way to elucidate topoisomerase II function and is also a promising first step towards a high-throughput drug screening assay to identify both new topoisomerase poisons as anticancer drug candidates and new topoisomerase catalytic inhibitors, which are becoming increasingly important for combined tumor therapies.

Experimental Section

Topoisomerase II and inhibitors: Purified bacteriophage T4 type II DNA topoisomerase (3.5 mg mL^{-1}; approx. 27.5 μM) was a gift from Kenneth N. Kreuzer (Duke University Medical Center, Durham, NC). The final concentration in the assays was 400 nM. Amsacrine hydrochloride (*m*-AMSA) was purchased from Sigma (Cat. no. A-9809) and dissolved in dimethyl sulfoxide (10 mg mL^{-1}; 23.25 mM). Dilutions of *m*-AMSA were in water. Novobiocin was from Sigma (Cat. no. N-1628) and was dissolved and diluted in water.

DNA substrates: The sequence of the double-stranded DNA substrate used in this work has been described previously.[24] Both 38-mer strands were custom-synthesized and HPLC-purified by IBA (Göttingen, Germany). Both oligos were ordered unmodified and, in addition, each was obtained labeled at its 5′-end with a fluorescence dye: Rhodamine green (Molecular Probes) or Cy5 (Amersham). Unlabeled and doubly labeled dsDNA substrates were prepared by annealing (600 nM) in cleavage reaction buffer without ATP. The thermal annealing program was 95 °C for 2 min, cooling to 23 °C with a slope of 1.2 °C min^{-1}, and holding at 23 °C for 10 min.

Topoisomerase cleavage reactions: Reactions were carried out in cleavage reaction buffer containing Tris-HCl (pH 7.6, 40 mM), KCl (60 mM), $MgCl_2$ (10 mM), EDTA (0.5 mM), DTT (0.5 mM), BSA (30 μg mL^{-1}), and ATP (0.5 mM). The concentration of the dsDNA substrate was 100 nM (50 nM unlabeled, 50 nM doubly labeled).

Samples were subjected to DC-FCCS immediately after mixing. Afterwards, they were incubated for 20 min at room temperature. Proteinase K was added to a final concentration of 0.05 mg mL^{-1}, and samples were incubated for an additional hour at room temperature and then subjected to DC-FCCS analysis again.

Dual-color fluorescence cross-correlation spectroscopy (DC-FCCS): DC-FCCS uses a confocal microscope setup to excite two spectrally distinct fluorophores in an overlapping volume element simultaneously. For both dyes, fluorescence emission is detected in separate channels: *i* and *j*. From each of the intensity traces, $F(t)$ autocorrelation functions are calculated. In addition, the intensity fluctuations of both channels are correlated with each other to give the cross-correlation function $G(\tau)$, according to Equation (1):

$$G_{ij}(\tau) = \frac{\langle \delta F_i(t) \delta F_j(t+\tau) \rangle}{\langle F_i(t) \rangle \langle F_j(t) \rangle} \quad (1)$$

The cross-correlation function $G_{ij}(\tau)$ is plotted against τ on a logarithmic scale (as, for example, in Figure 1). The mean number of singly labeled particles N_i and N_j present in the excitation volumes *i* and *j* is inversely proportional to the according amplitudes $G(0)_i$ and $G(0)_j$, respectively. The particle number N_{ij} of doubly labeled species can be derived from Equation (2):

$$N_{ij} = \frac{G_{ij}(0)}{G_i(0) \times G_j(0)} = G_{ij}(0) \times N_i \times N_j \quad (2)$$

In practice, the overlapping excitation volumes are not equal in size; because of different diffraction of the longer wavelength, the red focal volume element is most often larger than the green one. This leads to different values for N_i and N_j. It is evident that the number of doubly labeled species N_{ij} cannot exceed the particle number of the singly labeled species present in the smaller volume. If not all particles carry both labels, the relative fraction of doubly labeled species can therefore be calculated from Equation (3):

$$\frac{N_{ij}}{N_{(\text{lower particle number})}} = G_{ij}(0) \times N_{(\text{higher particle number})} = \frac{G_{ij}(0)}{G(0)_{(\text{lower autocorrelation amplitude})}} \quad (3)$$

From Equation (3) it is evident that the cross-correlation amplitude $G(0)_{ij}$ cannot exceed the lower autocorrelation amplitude, which in many cases is the one derived from the red channel.

Instrumentation and measurements: DC-FCCS analysis was carried out with the commercially available ConfoCor2 instrument (Zeiss, Germany). The fluorophores were excited with the 488 nm laser line of the argon ion laser and the HeNe 633 nm laser line. Correlation curves were averaged from 20 measurement of 10 s each, exported, and processed in Origin 7.0 (OriginLab, Northampton, MA, USA). Nonlinear least-squares fit routines with standard single-component models for auto- and cross-correlation analysis were used to obtain parameters such as amplitudes, $G(0)$, and diffusion times τ_d. As determined by the topoisomerase mechanism (see Results and Discussion), the signal range for decrease in cross-correlation amplitudes is from 1 to 0.5. The percentage of complexes is calculated by using Equation (4):

$$complex\ [\%] = 100 \times 2 \times \left(1 - \frac{G(0)_{CC,after} \times G(0)_{AC,before}}{G(0)_{AC,after} \times G(0)_{CC,before}}\right) \quad (4)$$

Here $G(0)_{CC}$ designates the cross-correlation amplitude and $G(0)_{AC}$ means the lower of the two autocorrelation amplitudes (most often the curve derived from the red channel, as the volume element from the red excitation light is larger due to wavelength-dependent diffraction reasons).

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