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Camptothecin-Stabilised Topoisomerase I–DNA Complexes in Leukaemia Cells Visualised and Quantified *In Situ* by the TARDIS Assay (trapped in agarose DNA immunostaining)

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ABSTRACT. We have shown that the TARDIS assay (trapped in agarose DNA immunostaining) can be used to detect DNA–topoisomerase I (topo I) cleavable complexes *in situ* in individual cells following treatment with topo I-targeting drugs. This assay is a modification of the assay for DNA–topoisomerase II (topo II) cleavable complexes (Willmore *et al.*, *Mol Pharmacol* **53:** 78–85, 1998). Drug-stabilised topo I–DNA complexes were detected *in situ* by topo I-specific primary antibodies and then visualised using fluorescein isothiocyanate conjugated second antibodies. Immunofluorescence was then quantified using a cooled slow-scan coupled device camera and image analysis procedures. Camptothecin (CPT) was shown to stabilise topo I–DNA cleavable complexes in whole cells in a dose-dependent manner in both CCRF-CEM and K562 cells and in lymphoblasts from an adult with newly diagnosed acute myeloid leukaemia treated *ex vivo* with CPT. In K562 cells, cleavable complexes were found to be maximal between 30 and 90 minutes continuous exposure of CPT, and approximately 78% of cleavable complexes formed in these cells were found to be reversed within 5 minutes of drug removal. It has also been shown that the immunofluorescence detected by the TARDIS assay was specific for topo I-targeting agents. Hence, the TARDIS assay provides a powerful tool to determine the levels of drug-stabilised cleavable complexes in whole cells and thereby aid in the understanding of the mechanism of interaction between topo I-targeting drugs and their target. BIOCHEM PHARMACOL **59**;6:629–638, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. camptothecin; topoisomerase I; cleavable complex; leukaemia cells; acute myeloid leukaemia; TARDIS assay

DNA topo I[‖] is the target for the CPT family of anticancer drugs which includes topotecan and irinotecan [1–3]. Topotecan and irinotecan are now in phase I and II clinical trials and have produced good responses in many human tumours either as single agents or in combination therapy [4]. Topo I is a monomeric protein, 100 kDa in size, which is encoded by a single gene on chromosome 20q11.2–13.1 [5, 6]. The enzyme is present in all nucleated cells in the nucleoplasm and is heavily concentrated in the nucleolus [7–9]. Topo I expression in human leukaemia HL-60 cells was found to be proliferation-dependent, with quiescent cells expressing lower levels of topo I than cycling cells [10]. Topo I catalyses an ATP-independent relaxation of both positively and negatively supercoiled DNA [11]. The enzyme affects DNA relaxation by transiently breaking a phosphodiester bond in a single DNA strand. Cleavage of the DNA is achieved by forming a topo I–DNA covalent intermediate between a tyrosyl residue and the 3′ end of the DNA. This intermediate is often referred to as the 'cleavable complex'. The cleaved DNA strand is then rotated around the unbroken strand. Reversal of the transesterification reaction then restores the phosphodiester bond and the enzyme is released [12, 13]. Topo I has been shown to provide the relaxation activity required for both DNA replication [14–16] and transcription [17, 18]. Drugs such as CPT, topotecan, and irinotecan interact with topo I and cause stabilisation of the 'cleavable complex' [19, 20], primarily by inhibiting the rate of DNA religation by the enzyme [21]. These CPT-stabilised cleavable complexes

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‖ *Abbreviations:* AML, acute myeloid leukaemia; CPT, camptothecin; FITC, fluorescein isothiocyanate; TARDIS, trapped in agarose DNA immunostaining; topo, topoisomerase; PMSF, phenylmethanesulphonyl fluoride; and DTT, dithiothreitol.

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have been shown to be reversible upon removal of the drug [22, 23]. However, it has been proposed that persistent CPT-stabilised cleavable complexes lead to DNA damage through collision with an active replication fork, resulting in fork arrest and fork breakage. This in turn triggers cellular events which ultimately bring about cell death [24, 25]. The strong S-phase specificity of CPT supports this model [26]. In addition, studies using replication inhibitors such as aphidicolin have shown that transient inhibition of replication during CPT treatment can abrogate CPT toxicity [27, 28]. Another mechanism of cell killing is also thought to exist which can be distinguished from the fork collision mechanism because it is unaffected by aphidicolin. This causes G_2 arrest probably by down-regulation of $p34^{cdc2}$ kinase [29].

Development of resistance is a major problem in producing successful anticancer therapy. Studies have shown that structural mutations of topo I can confer a CPT-resistant phenotype without affecting topo I enzyme activity [30, 31]. In addition, both altered activities and decreased levels of topo I have been shown to be potential mechanisms for drug resistance [32–34]. Acquired resistance may also be caused by changes in the downstream pathway from topo I-induced damage, e.g. enhanced DNA repair [35] or perhaps decreased conversion of CPT-induced single-stranded breaks into more stable irreparable double-stranded breaks [36].

Here, we report that topo I cleavable complexes can be quantified in individual cells by the application of the TARDIS assay. Briefly, CPT-treated cells are embedded in agarose and lysed to remove the cell membrane and soluble proteins. This process leaves the DNA from each cell trapped in the agarose along with any associated drug-stabilised cleavable complexes. The covalently bound topo I can then be detected *in situ* by anti-topo I antibodies and visualised by the use of FITC-conjugated second antibodies and fluorescence microscopy. Utilising this method, we show that topo I forms cleavable complexes in both CCRF-CEM and K562 human leukaemia cells in a dose dependent manner in whole cells following CPT treatment. In addition, we show that the TARDIS method can detect cleavable complexes in lymphoblasts from a patient with AML treated *ex vivo* with CPT.

MATERIALS AND METHODS

Cell Culture

K562 human chronic myelogenous leukaemia (CML) cells and CCRF-CEM human acute lymphoblastic leukaemia (ALL) cells were grown as suspension cultures in RPMI-1640 supplemented with 3 mM L-glutamine, 10% foetal bovine serum (FBS), penicillin (50 units/mL) and streptomycin (50 μg/mL) at 37° (in a humidified atmosphere containing 5% CO_2). Both cell lines were maintained at a density between 1×10^5 and 1×10^6 cells/mL. The cell lines were tested for contamination with mycoplasma and were found to be negative.

Drug Treatments and Irradiation

CPT (0.7 mg/mL stock dissolved in DMSO and stored at −20°) was purchased from ICN. Exponentially growing cells ($\sim 5 \times 10^5$) were exposed to CPT for either 30 min or 2 hr depending upon the cell line used. Etoposide (1 mg/mL stock dissolved in methanol and stored at −20°) was purchased from Sigma. Exponentially growing cells ($\sim 5 \times 10^5$) were exposed to etoposide for 2 hr. Following drug exposure, cells were washed twice by centrifugation (400 × *g*, 3 min) in cold PBS. For irradiation experiments, cells were irradiated in PBS (0°) at 4 Gy/min using a ^{137}Cs source (Gammacell 1000; Nordion International, Inc.). In order to study the reversibility of cleavable complexes, drug-treated cells were washed by centrifugation and incubated in fresh medium for various time points before being finally washed and resuspended in fresh PBS (0°).

Cytotoxicity Assay

Drug cytotoxicity was assayed by seeding cells onto 96-well plates at a density of $\sim 1 \times 10^5$/mL. After 48-hr incubation, drug was added (at the indicated concentrations) to triplicate wells for each drug dose. After 30-min incubation with drug, cells were washed twice with PBS and then resuspended in fresh medium. After a further 96-hr incubation, the trypan blue-excluding cells were counted and the results expressed as described previously [37] as a percentage of growth compared with controls.

Agarose Embedding and Staining

The method for precoating microscope slides and embedding cells in agarose was as described previously [38]. Briefly, a solution of low melting point agarose (SeaPrep ultra-low gelling, FMC BioProducts; 0.5% w/v in H_2O) was spread thinly onto microscope slides (Western Laboratory Service Ltd.) and dried at room temperature to form an adherent coating. Drug-exposed cells resuspended in PBS (0°) were warmed to 37° and immediately mixed with an equal volume of agarose solution (SeaPrep ultra-low gelling, FMC BioProducts; 2% w/v in PBS) which had been melted and kept at 37°, after which this mixture was spread evenly across the precoated slides. These were placed immediately on a cold surface (0°) in order to solidify the agarose and minimise evaporation of water. The slides were then immersed in lysis buffer (1% w/v SDS, 80 mM phosphate buffer pH 6.8, 10 mM EDTA, 2 μg/mL pepstatin, 2 μg/mL leupeptin, 1 mM PMSF, 1 mM benzamidine, and 1 mM DTT) for 30 min at room temperature. At this stage, the slides could be stored at −20° in PBS containing 10% glycerol without loss of signal. Next, slides were immersed in 1 M NaCl supplemented with protease inhibitors (2 μg/mL pepstatin, 2 μg/mL leupeptin, 1 mM PMSF, 1 mM benzamidine, and 1 mM DTT) for 30 min at room temperature. After washing three times for 5 min each in PBS, the slides were exposed to primary antiserum (2012)

for 2 hr in a humidified atmosphere. 2012 was a polyclonal human antibody to human topo I from Topogen, diluted 1 in 1000 in PBS containing 0.1% v/v Tween 20 (PBST) and 1% w/v BSA. The slides were then washed three times (5 min) in PBST. The slides were then exposed to the FITC-conjugated goat anti-human immunoglobulin (Ig) G (Fab-specific) second antibody for 2 hr (from Sigma), diluted 1 in 50 in PBST containing 1% w/v BSA before two 5-min washes in PBST and one final wash (20 min or overnight at 4°) in PBS containing protease inhibitors (2 μg/mL pepstatin, 2 μg/mL leupeptin, 1 mM PMSF, 1 mM benzamidine, and 1 mM DTT). Slides were then stained with Hoechst 33258 (10 μmol/L in PBS) for 5 min and coverslips placed carefully onto each slide.

Quantitative Immunofluoresent Microscopy and Image Analysis

The method has been described in detail previously [39]. The blue (Hoechst-stained DNA) fluorescence and the green (FITC-stained topo I) immunofluorescence were visualised separately using an epifluorescence microscope (Olympus BH2-RFCA) and appropriate sets of optical filters (Omega Optical, Inc.). Fluorescence images for quantitative analysis were obtained using a ×10 objective and were captured using a cooled slow-scan charge-coupled device (CCD) camera (Astrocam Ltd.). Images were digitised at an accuracy of 16 bits per pixel. DNA in a particular field of view was focused only under blue fluorescence, and fluorescence was captured using a 5-sec exposure. Then, an image of the green immunofluorescence for the same field of view was captured (40-sec exposure). Eight pairs of images per drug dose of randomised fields of view were captured from replicate slides giving approximately 150–200 cells per dose.

Initially, all images were corrected for stray light and camera background. All sample images were also subjected to shade correction to compensate for variation in intensity of illumination and for non-uniformities in light transmission as described previously [39]. The corrected images for Hoechst were used to define the areas occupied by the DNA for each cell. This was achieved by creating a binary image using a grey-scale threshold which was set at a level so that all objects could be demarcated. Each binary image was first refined by 'erosion' and 'reconstruction' to remove any non-specific 'specks' of fluorescence between the cells. The background level of fluorescence for each image was calculated as the mean fluorescence intensity of all the pixels lying between the areas occupied by DNA. This value was then subtracted from each pixel and a corrected grey-scale image produced. Integrated fluorescence intensities for Hoechst and FITC images are the sums of the grey-scale values of all the pixels within each object. Valid objects were those defined by the computer software that did not touch the edge of the image. Objects consisting of more than one cell were excluded from this analysis.

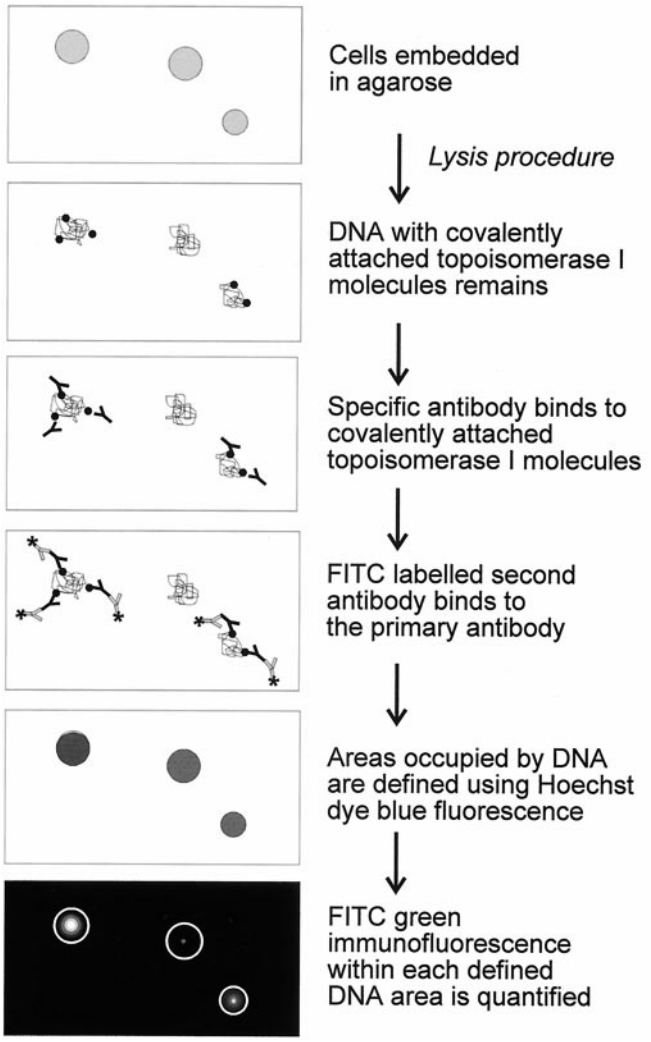


FIG. 1. Schematic diagram of the 'TARDIS' method to detect drug-stabilised DNA–topo I cleavable complexes.

RESULTS

Immunofluorescent Staining of Topo I Drug-Stabilised Cleavable Complexes in CCRF-CEM Cells

CCRF-CEM cells were exposed to a range of concentrations of CPT for 2 hr and then immediately analysed using the assay illustrated in Fig. 1 to detect drug-stabilised topo I cleavable complexes in individual cells. First, cells were embedded in agarose on microscope slides and then lysed to remove the cell membrane and soluble proteins. Extraction with 1 M NaCl was then used to remove nuclear proteins. This process leaves the DNA from each cell trapped in agarose along with any drug-stabilised cleavable complexes associated with the DNA. The covalently bound topo I can then be detected *in situ* by immunofluorescence by staining with an anti-topo I primary antibody followed by an FITC-conjugated second antibody.

Figure 2 illustrates five pairs of images typical of those seen after staining with the primary antibody. Figure 2, a, c, e, g, and i show DNA-specific blue Hoechst staining of the cells for 0, 0.1, 1.0, 10, and 100 μM CPT, respectively.

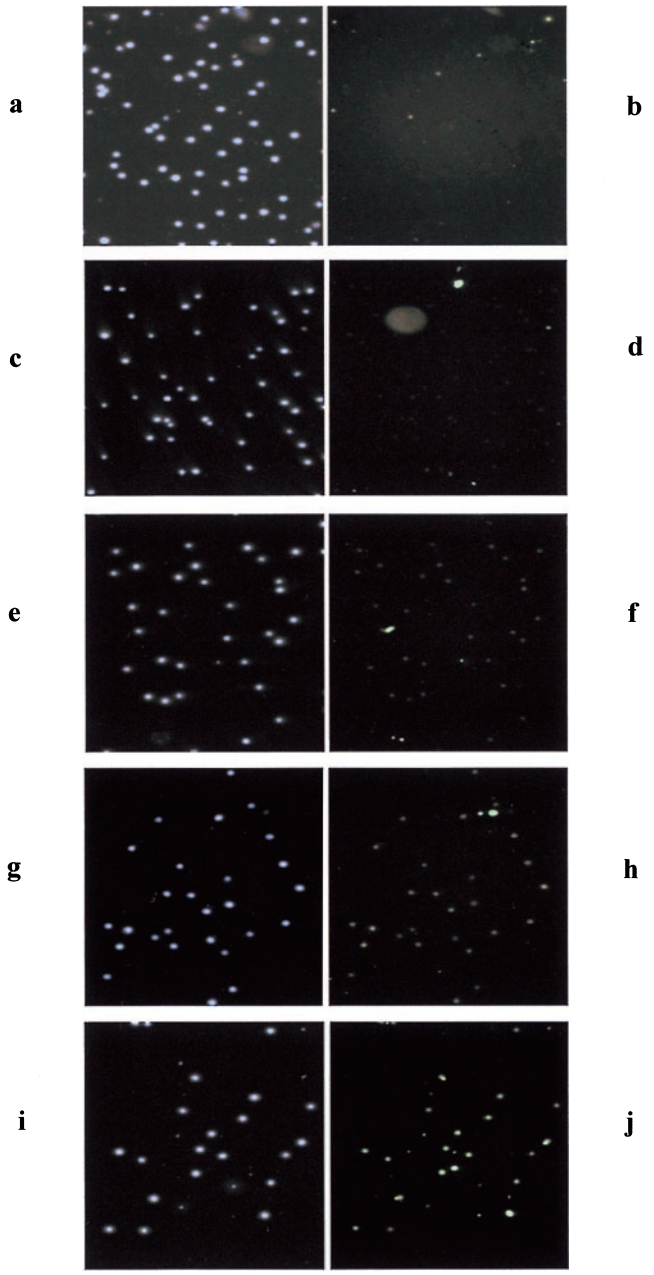


FIG. 2. Fluorescence of CPT-treated CCRF-CEM cells. Cells were exposed to 0 (a, b), 0.1 (c, d), 1.0 (e, f), 10 (g, h), and 100 μM (i, j) CPT for 2 hr. The cells were then embedded in 2% low melting point agarose on microscope slides and stained with primary antiserum and a secondary FITC-conjugated antibody. Images a, c, e, g, and i illustrate the Hoechst-stained DNA (blue), whereas images b, d, f, h, and j illustrate the corresponding FITC-stained (green) immunofluorescence, the result of stabilised cleavable complexes. (All images were typical of those seen in replicate experiments).

Figure 2, b, d, f, h, and j show the corresponding FITC-stained (green) immunofluorescence. There was no detectable immunofluorescence associated with the DNA in untreated cells (Fig. 2b), however at 0.1 μM CPT, very low levels of immunofluorescence were just detectable (Fig. 2d). Immunofluorescence was easily detectable in CCRF-CEM cells exposed to 1, 10, and 100 μM. This staining intensity was dose-dependent, and the level of detectable immunofluorescence increased with CPT concentration. No FITC staining was observed in drug-treated cells when the primary antibody was omitted, nor did CPT alone produce fluorescence in the assay system (data not shown).

Digital images of Hoechst DNA fluorescence and FITC immunofluorescence were then subjected to the analysis procedures to quantify the levels associated with the DNA from each individual cell (see Materials and Methods). Detailed results for a typical experiment are shown in Fig. 3. Figure 3, a and b shows scattergrams in which each cell analysed is depicted as a dot. As the distributions of integrated fluorescence intensities for FITC immunofluorescence and Hoechst fluorescence were mostly non-Gaussian as determined by the Kolmogorov–Smirnov test (data not shown), median fluorescence values were calculated for each dose and are shown in Fig. 3, c and d. Low levels of FITC immunofluorescence were associated with untreated CCRF-CEM cells (Fig. 3d). After 0.1 μM CPT, levels of FITC immunofluorescence were significantly higher than in the control cells ($P < 0.0001$, Mann–Whitney test). Further significant increases ($P < 0.0001$, Mann–Whitney test) in FITC immunofluorescence were seen after 1.0, 10, and 100 μM CPT compared to the control cells. The Hoechst fluorescence levels did not change significantly from the control cells with increasing concentration of CPT (Fig. 3c). Comparable significant increases in immunofluorescence were observed in each of several replicate experiments (data not shown).

Immunofluorescent Staining of Topo I Drug-Stabilised Cleavable Complexes in K562 Cells

K562 cells were exposed to 10 μM CPT for 0.25, 0.5, 1, 1.5, 2, 3, and 5 hr. They were then processed as described previously and levels of Hoechst fluorescence and FITC immunofluorescence in individual cells were quantified (Fig. 4). Levels of Hoechst fluorescence in CPT-treated cells did not significantly differ from the control cells (Fig. 4a). Figure 4b illustrates that after 15-min exposure to CPT, significantly higher levels of integrated fluorescence were detected as compared to the control cells ($P < 0.0001$ Mann–Whitney test). There was a further significant increase in fluorescence values after 30 min as compared to 15-min exposure ($P < 0.0001$ Mann–Whitney test). After 1- and 1.5-hr constant exposure to CPT, the levels of integrated fluorescence remained similar to that seen after 30-min exposure. However, after 2-hr constant exposure to 10 μM CPT, a significant decrease in the levels of cleavable complexes was seen ($P < 0.0001$, Mann–Whitney test). A further decrease in FITC-integrated fluorescence levels was seen after 5 hr ($P < 0.0001$, Mann–Whitney test). Hence, a 30-min drug exposure was utilised in all future experiments using the cell line K562. Figure 5 illustrates the results of three independent experiments in which K562 cells were exposed to varying concentrations of CPT for 30 min. Figure 5a shows no significant changes in the levels of Hoechst fluorescence with increasing doses of CPT. Figure 5b shows a dose-dependent increase in

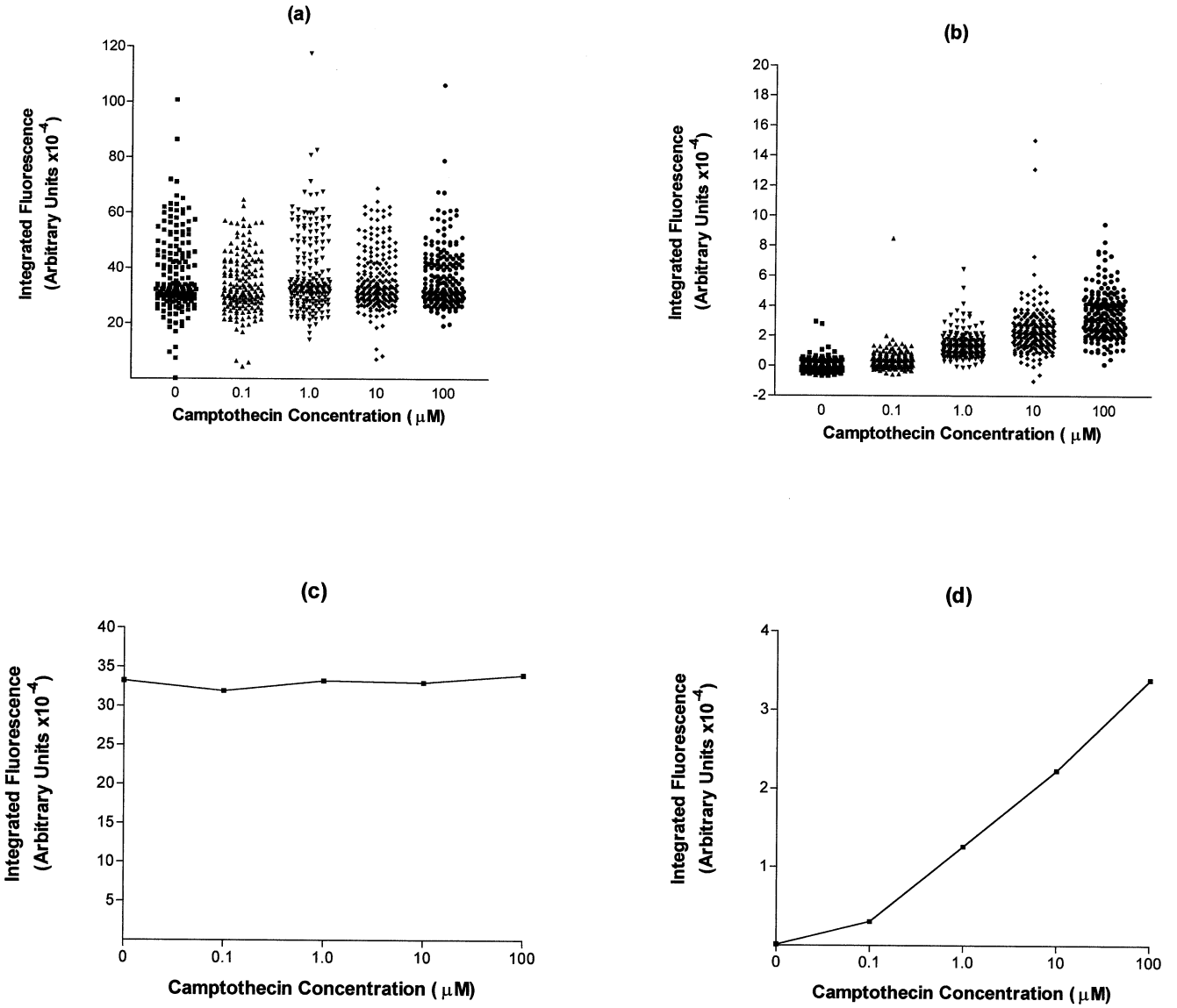


FIG. 3. Dose-dependent nature of fluorescence in CCRF-CEM cells following CPT treatment. Cells were exposed to 0, 0.1, 1.0, 10, and 100 μM CPT for 2 hr. Slides were then prepared and stained as described in the text. (a) and (b) represent scattergrams of Hoechst fluorescence and FITC immunofluorescence, respectively. Plots of median fluorescence values are also shown for Hoechst (c) and FITC (d). The data shown are the result of one experiment.

FITC immunofluorescence levels. The FITC immunofluorescence levels for 0.1, 1.0, and 10 μM were significantly higher than for the control cells in each individual experiment ($P < 0.0001$, Mann–Whitney test). The cytotoxic effect of CPT on K562 cells following a 30-min exposure as defined by the IC_{50} was found to be 0.04 ± 0.01 μM. After 10 μM CPT, there were no detectable surviving cells.

To determine whether the increase in immunofluorescence was specific for topo I-targeting agents, a number of controls were performed. K562 cells were exposed to a range of concentrations of CPT for 30 min, while other aliquots of the cells were exposed to 10 μM etoposide for 30 min or to 10 Gy of γ irradiation. Slides were then prepared and stained as before. Figure 6 shows the expected increasing levels of FITC immunofluorescence with increasing doses of CPT, but K562 cells exposed to γ irradiation yielded lower levels of fluorescence than the control cells ($P = 0.0036$, Mann–Whitney test). K562 cells exposed to 10 μM etoposide yielded an immunofluorescence level that was not significantly different from that of the control cells ($P = 0.7027$, Mann–Whitney test).

Topo I cleavable complexes are known to reverse following drug removal. Therefore, the time-dependent reversibility of CPT–topo I cleavable complexes in K562 cells was examined. The results of three independent experiments are shown in Fig. 7a. K562 cells were exposed to 1 μM CPT for 30 min. The medium-containing drug was then removed from the cells, and the cells were then incubated in drug-free medium for various times prior to slide preparation. At time zero, at which point the K562 cells were prepared immediately following exposure to CPT, high levels of FITC-integrated fluorescence were detected. After 5 min in drug-free medium, the levels of FITC immunoflu-

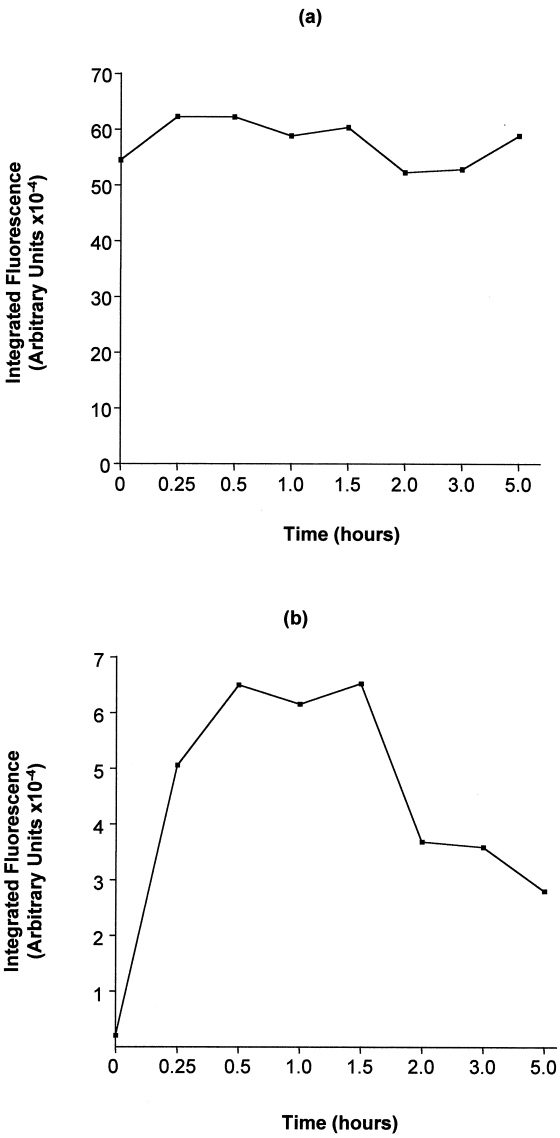


FIG. 4. Time-dependent formation of CPT-stabilised cleavable complexes in K562 cells. Cells were continually exposed to 10 μM CPT for various times. Slides were then prepared and stained as described in the text. The plot illustrates the median Hoechst fluorescence values (a) and the median FITC immunofluorescence values (b) obtained from a single experiment.

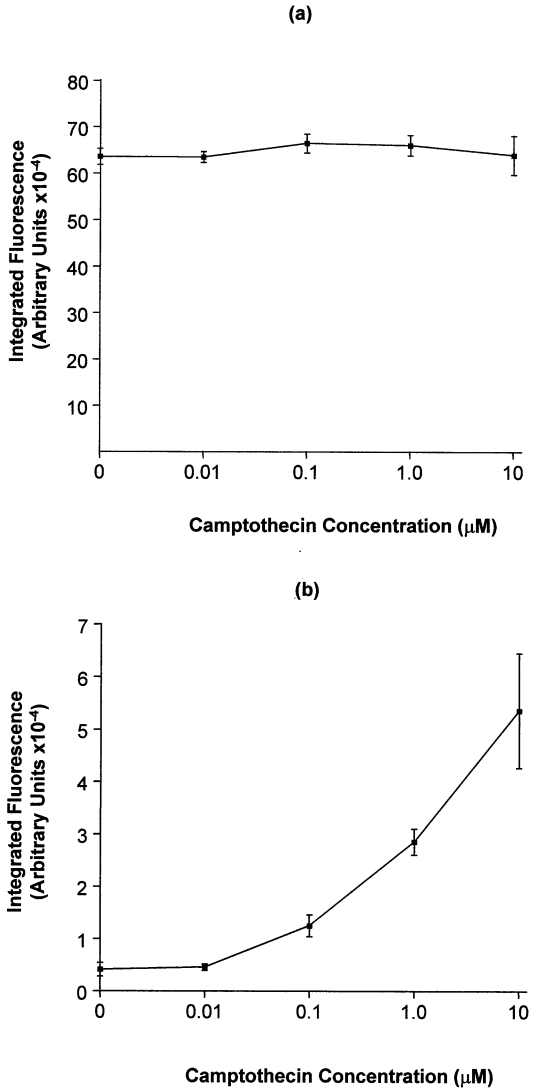


FIG. 5. Quantitation of CPT-stabilised cleavable complexes in K562 cells. Cells were exposed to varying concentrations of drug for 30 min. Slides were then prepared and stained as described in the text. Plots illustrate means and standard errors calculated from median Hoechst fluorescence values (a) and FITC immunofluorescence values (b) obtained from three independent experiments.

orescence had decreased significantly ($P < 0.001$, repeated measures ANOVA) to ~22% of that seen in K562 cells processed immediately following drug treatment. A further decrease in FITC immunofluorescence levels was seen after 10, 15, and 20 min, however these were not significantly different from the levels after 5 min ($P > 0.05$, repeated measures ANOVA). This indicated that after 5-min incubation in drug-free medium, the majority of the topo I drug-stabilised cleavable complexes formed following 30-min incubation with 1 μM CPT had disappeared. From the results of each individual experiment in which each cell analysed was depicted as a dot, an example of which is shown in Fig. 7b, it can be seen that the decrease in FITC levels after 5-min incubation in drug-free medium occurred in the majority of the cell population.

Immunofluorescence Staining of Topo I Drug-Stabilised Cleavable Complexes in AML Lymphoblasts

Lymphoblasts were obtained from an adult with newly diagnosed AML. These cells were then exposed to varying concentrations of CPT for 2 hr *ex vivo* (0, 0.1, 1.0, 10, and 100 μM). CCRF-CEM cells were simultaneously exposed to 0, 1.0, and 10 μM CPT for 2 hr as a series of positive controls. Figure 8 shows that FITC immunofluorescence in AML blasts exposed to 1.0 μM CPT for 2 hr was significantly higher than for the control cells ($P < 0.0001$, Mann–Whitney test). Further significant increases were seen in AML blasts exposed to 10 and 100 μM CPT ($P < 0.0001$ and $P < 0.0001$, respectively, Mann–Whitney test). The expected dose-dependent increase in FITC immunofluorescence levels was seen in the CCRF-CEM cells (data not shown).

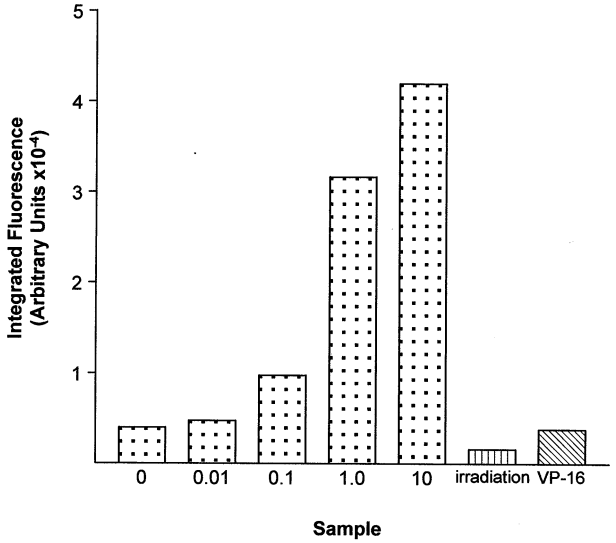


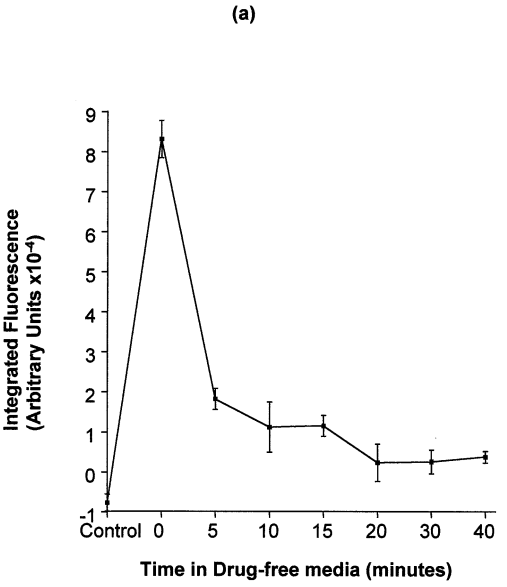
FIG. 6. Specificity of complex recognition. K562 cells were exposed to 0, 0.01, 0.1, 1.0, and 10 μM CPT for 30 min. Another aliquot of K562 cells from the same culture was exposed to 10 Gy of γ irradiation and another aliquot was exposed to 10 μM etoposide (VP-16) for 30 min. Slides were prepared and stained as described in the text. The plot illustrates the median FITC immunofluorescence values obtained from a single experiment.

DISCUSSION

This report describes the use of the TARDIS assay to detect CPT-induced topo I cleavable complexes in individual cells. The results also demonstrate that topo I forms cleavable complexes in CCRF-CEM and K562 cells in a dose-dependent manner, the assay signal becoming significant in both cell lines and at 0.1 μM CPT, (Figs. 3 and 5, respectively). The IC_{50} value for growth inhibition by CPT on K562 cells was found to be 0.04 ± 0.01 μM.

Alkaline elution experiments have revealed an approximate 5-fold increase in DNA–protein cross-links in K562 cells exposed to 1.0 μM CPT for 30 min compared with K562 cells exposed to 0.01 μM CPT for 30 min [40]. Similarly, the TARDIS data from three independent experiments in which K562 cells were exposed to 0.01 and 1.0 μM CPT for 30 min showed an approximate 6-fold increase in assay signal (Fig. 5).

In the K562 cell line during a continuous exposure of 10 μM CPT, the formation of CPT–topo I-stabilised cleavable complexes showed an initial increase with time, with maximal levels being formed after 30-min exposure. After 2-hr exposure, a significant decrease in the levels of cleavable complexes was seen (Fig. 4). This time-dependent decrease in levels of cleavable complexes has been noted previously [8, 23, 41, 42]. CPT could either be metabolised to an inactive compound, or some other intracellular events following exposure to CPT may occur which may modify cellular responses to the drug in a time-dependent manner. Examples of such events would be a decrease in whole-cell topo I protein level [23] or ubiquination and destruction of topo I cleavable complexes [43]. It has also been reported that an enzymatic activity that



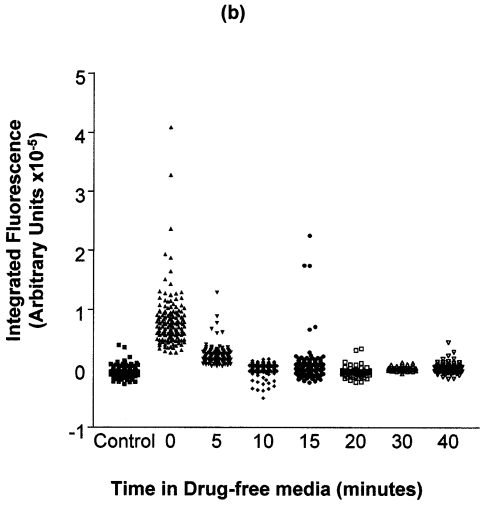


FIG. 7. Reversibility of drug-stabilised topo I cleavable complexes. K562 cells were exposed to 1 μM CPT for 30 min. Slides were then prepared immediately after drug was washed out and after cells were incubated in fresh medium for 5, 10, 15, 20, 30, or 40 min. Slides were prepared and stained as described in the text. Plots illustrate the means and standard errors for median FITC immunofluorescence values obtained from three independent experiments (a) and a scattergram of FITC immunofluorescence values obtained from a single experiment (b). (Hoechst data not shown).

excises covalent 3′ DNA–protein complexes exists [44]. Such a repair mechanism could account for the decreased levels of cleavable complexes despite persistent levels of CPT.

The levels of FITC immunofluorescence detected in K562 cells were greater than those detected in CCRF-CEM cells at equivalent concentrations of CPT. This may have been due to the use of suboptimal exposure time, resulting in decreased levels of cleavable complexes in CCRF-CEM cells. In addition, quantitative or qualitative differences in

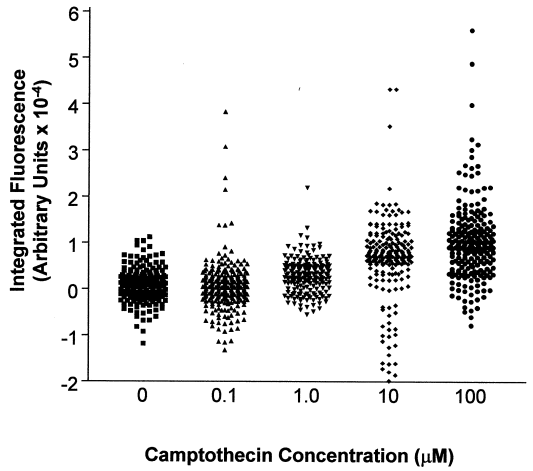


FIG. 8. Immunofluorescent quantitation of CPT-stabilised cleavable complexes in individual AML cells. Lymphoblasts from an adult with newly diagnosed AML were exposed to varying concentrations of drug in RPMI-1640 for 2 hr. Slides were then prepared and stained as described in the text. The plot illustrates a scattergram of FITC immunofluorescence values obtained from a single experiment. (Hoechst data not shown).

topo I between the two cells lines may exist. Some studies indicate that cells containing more topo I polypeptide form more topo I–DNA cleavable complexes [45]. However this is not true for all cell lines. It has been demonstrated that no relationship existed between topo I expression and topo I-induced cleavable complexes in 7 colon carcinoma cell lines [46]. Other cellular events such as drug uptake may also account for such differences between cell lines.

Several observations confirmed that the immunofluorescence measured in this assay was specific for topo I cleavable complexes. First, the primary antibody did not bind to DNA breaks alone produced by γ irradiation (Fig. 6). Second, the primary antibody did not bind to another closely related protein-linked strand break, i.e. etoposide-stabilised topo II cleavable complexes (Fig. 6). Third, it is well documented that topo I cleavable complexes are readily reversible upon removal of drug [22, 23]. The results shown here indicate that after only 5-min incubation in drug-free medium, ~78% of cleavable complexes present immediately after a continuous 30-min exposure to 1 μM CPT had disappeared (Fig. 7a).

In lymphoblasts from an adult with newly diagnosed AML treated *ex vivo* with varying concentrations of CPT, an increase in FITC immunofluorescence values was seen with increasing concentrations of CPT. These became highly significant at 1 μM CPT. AML blasts treated with 100 μM CPT yielded similar levels of FITC immunofluorescence as did 1 μM CPT in CCRF-CEM cells. This may have been due to the use of suboptimal exposure time, differences in drug uptake, and/or topo I protein levels. Possible differences in protein levels may be cell cycle-related, as topo I has been shown to be more highly expressed during logarithmic growth [10, 47].

Various methods have been employed to quantify the amount of topo I covalently bound to DNA, for example SDS–KCl precipitation [8, 23], alkaline elution using non-deproteinising conditions [40], and the ICE bioassay [48]. The TARDIS assay described in this paper utilises a topo I-specific antibody and has been shown to specifically detect topo I-stabilised cleavable complexes in a dose-dependent manner. The TARDIS assay has a number advantages over previous methods in that it can be used to quantify topo I-stabilised cleavable complexes *in situ* in individual cells, which may reveal cell-to-cell heterogenity in forming cleavable complexes. In addition, relatively small numbers of cells are required for the TARDIS assay, making it a more feasible assay when dealing with clinical samples where sample material may be limited.

We have shown that the TARDIS assay can be used to quantify drug-stabilised topo I cleavable complexes in individual cells and that CPT-stabilised cleavable complexes form in a dose-dependent manner in human leukaemia CCRF-CEM and K562 cells. In addition, the technique has been shown to be capable of detecting cleavable complexes in lymphoblasts from a patient with AML treated *ex vivo* with CPT. Topo I drug-stabilised cleavable complexes in K562 cells have also been shown to be time-dependent in the continuous presence of drug and were following drug removal. If this time-dependent decrease in cleavable complex formation were found to cause a reduction in cytotoxicity and this proved to be a common response in human tumours *in vivo*, then this may have important implications for dosing schedules [23].

The TARDIS assay is a powerful tool with which to elucidate the effect of drug combinations and drug scheduling on stabilisation of cleavable complexes in individual cells. Understanding the efficacy of the CPT family of drugs over time is crucial for designing effective dosing strategies either as a single agent or in combination with other drugs. Therefore, in combination with pharmacokinetic data and data generated about events downstream of cleavable complex formation, use of the TARDIS assay will provide a wider understanding of action of topo I-targeting drugs. In addition, this assay in combination with the topo II α and β TARDIS assay [38] will be used to identify and study dual topo I and topo II inhibitors.

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