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# A Flow Cytometry-Based Method for a High-Throughput Analysis of Drug-Stabilized Topoisomerase II Cleavage Complexes in Human Cells

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• **Abstract**

Topoisomerase II (Top2) is an important target for anticancer therapy. A variety of drugs that poison Top2, including several epipodophyllotoxins, anthracyclines, and anthracenediones, are widely used in the clinic for both hematologic and solid tumors. The poisoning of Top2 involves the formation of a reaction intermediate Top2-DNA, termed Top2 cleavage complex (Top2cc), which is persistent in the presence of the drug and involves a 5′ end of DNA covalently bound to a tyrosine from the enzyme through a phosphodiester group. Drug-induced Top2cc leads to Top2 linked-DNA breaks which are the major responsible for their cytotoxicity. While biochemical detection is very laborious, quantification of drug-induced Top2cc by immunofluorescence-based microscopy techniques is time consuming and requires extensive image segmentation for the analysis of a small population of cells. Here, we developed a flow cytometry-based method for the analysis of drug-induced Top2cc. This method allows a rapid analysis of a high number of cells in their cell cycle phase context. Moreover, it can be applied to almost any human cell type, including clinical samples. The methodology is useful for a high-throughput analysis of drugs that poison Top2, allowing not just the discrimination of the Top2 isoform that is targeted but also to track its removal. 

• **Key terms**

flow cytometry; topoisomerase II; Top2 poison; high throughput analysis

**TOPOISOMERASE** II (Top2) is a dimeric enzyme that catalyzes topological changes in the DNA, thus being essential in several metabolic processes of the genome of eukaryotic cells (1,2). In mammals, there have been characterized two variants of Top2, $\alpha$ and $\beta$ isoforms, showing different activities and regulation of their expression. While Top2$\alpha$ expression and activity increase throughout the cell cycle, Top2$\beta$ remains at similar levels (3,4). Top2$\alpha$ is essential for cell proliferation, participating in several processes such as replication, transcription, and chromosome condensation and segregation (5). In contrast, Top2$\beta$ is dispensable for cell proliferation, but contributes to transcription and is required during development (6,7). Top2 acts by nicking both strands of the DNA to allow the passage through it of other double-stranded segment of DNA from the same or a different molecule. Following the breakage of the double-stranded DNA, each monomer of Top2 remains covalently linked to each 5′ terminus of DNA by a transient phosphotyrosyl linkage, a reaction intermediate termed cleavage complex (Top2cc) (8,9). Certain natural compounds, spontaneous DNA lesions and several chemotherapeutic agents can stabilize the Top2cc increasing their half-life (10–12). These agents interfere with the breakage-reunion reaction that leads to a reversible DNA damage, which becomes irreversible

during processes such as transcription and replication (13). Among the clinically relevant agents that poison Top2 are the epipodophyllotoxin etoposide (ETO), the anthracycline idarubicin (IDA), and the anthracenedione mitoxantrone (MTX). Cumulative evidence suggests a tight association between the persistent DNA damage resulting from stabilizing Top2cc and the cytotoxicity of these drugs (14,15).

The removal of the protein covalently bound to DNA is an important task for the repair of Top2-mediated damage. So far, the evidence points to different mechanisms for its removal including activities of endonucleolytic cleavage (16), enzyme-mediated hydrolysis of the phosphotyrosyl linkage (17,18), and proteolysis by a proteasome-dependent pathway (8). In any case, their relative contribution during different cellular processes is not clear yet.

There are several methods for assaying in vivo drug stabilized-Top2cc and its removal. Widely used methods, such as the immunocomplex of enzyme (ICE) assay, involves the physical separation of Top2 adducts but requires large samples and an extensive cesium chloride gradient ultracentrifugation; thus, it is unsuitable for high-throughput analysis. The trapped in agarose DNA immunostaining (TARDIS) assay is an immunofluorescence-based method for the detection of Top2cc from cells immobilized in agarose, which is limited by significant handling of the sample and the restricted number of cells that can be analyzed. Other immunofluorescence-based method is the differential retention of Top2 (DRT) assay. This method utilizes a high-salt and detergent extraction step for removing non-covalently bound Top2. While this method is very informative for the sub-nuclear localization of Top2cc (19), it is incompatible with non-adherent growing cells.

Based on these limitations, we developed a quick and easy flow cytometry-based method for a high-throughput analysis of Top2cc in almost any mammalian cell type. This method utilizes a combination of heparin and detergent for the extraction of unbound or weakly-bound Top2 from DNA, and a fluorescent DNA intercalating dye which enables the simultaneous analysis of the cell cycle by flow cytometry. By making use of the proteasome inhibitor bortezomib, we also show the potential of this method to track drug-stabilized Top2cc removal and identify factors affecting this process.

## Materials and Methods

### Cell Lines and Cultures

The human promyeloblast HL-60 cells (ATCC, #CCL-240), the acute myeloid leukemia K-562 cell line (ATCC, #CCL-243) and the cervix adenocarcinoma HeLa (ATCC, #CCL-2) cell line were grown in RPMI 1640 supplemented with 10% FBS and 2 mM l-glutamine and antibiotics. The human neuroblastoma SH-SY5Y cell line (ATCC, #CRL-2266) was grown in DMEM supplemented with 10% FBS and 2 mM l-glutamine and antibiotics. Human peripheral blood mononuclear cells from healthy donors were isolated through centrifugation on a Ficoll-Paque Plus (GE Healthcare Bio-Sciences) density gradient. Mononuclear cells were washed with PBS. Adherent cells (Monocytes) were separated by plastic adherence by incubating at 37°C by 45 min. Then, peripheral blood lymphocytes (PBL) were stimulated with both phytohemagglutinin and pokeweed mitogen for 48 h in RPMI-1640 media supplemented with 15% FBS and 2 mM l-glutamine and antibiotics. All the experiments were performed between July 2015 and February 2016.

### Reagents and Antibodies

Etoposide (ETO; CAS No. 33419–42-0; Sigma) and Bortezomib (Btz; CAS No. 179324–69-7, Santa Cruz Biotechnology) were diluted in DMSO. Idarubicin (IDA; CAS No. 58957–92-9; Pharmacia & Upjohn) and Mitoxantrone (MTX; CAS No. 70476–82-3; Sigma) were diluted in bidistilled water. Unfractionated heparin (5,000 U/ml) was from Fada Pharma.

The primary antibodies used in this study were: rabbit anti-Top2a (Santa Cruz Biotechnology H-231; 1:200), mouse anti-Top2$\beta$ (Santa Cruz Biotechnology H-8; 1:200). The secondary antibodies were: Alexa Fluor 488-conjugated goat anti-rabbit (Life Technologies; 1:100); or DyLight 488-conjugated goat anti-mouse (Thermo Scientific; 1:100).

### Sample Preparation for Flow Cytometry

Non-adherent growing cell lines were seeded at a density of $1 \times 10^6$ cells in 35 mm culture dishes 48 h before the experiments. PBL cultures were seeded 48 h before the experiments. Adherent growing cells were seeded at a 60–70% of confluence.

The cells were treated with ETO by 1 h and harvested or after washing twice with 1× phosphate-buffered saline (PBS) the cells were cultured in ETO-free media to allow its recover for an additional 30–60 min. Harvested cells were maintained in cold-PBS on ice. Cells were then centrifuged and resuspended in 500 µl of cold PHEM buffer (65 mM Pipes, 30 mM HEPES, 10 mM EGTA, 2 mM $Mg_2Cl$, pH 6.9) containing 2 mM PMSF. Then, it was added an equal volume of PHEM buffer containing 1% Triton X-100 and heparin (100U). Alternatively, it was added the PHEM buffer but containing 1% Triton X-100 and 700 mM NaCl (350 mM NaCl final concentration) for salt-extraction instead of heparin for comparison purposes. The samples were then extracted by mixing for 5 min under a rotating microtube mixer (Bioelec) by gentle inversions at 4°C. Salt-extracted samples were mixed by 2 min under the same conditions. The fixation was performed by adding 325 µl of 4% paraformaldehyde per tube and incubated at room temperature by 30 min. After washing twice with PBS, the samples were resuspended in 50 µl of blocking buffer (3% BSA, 0.5% Triton X-100 in PBS) and incubated for 1 h. Subsequently, they were incubated with the primary antibody diluted in blocking buffer for 2 h at room temperature. The samples were then washed in PBS and incubated with 50 µl of the appropriate secondary antibody diluted in blocking buffer for 1 h. After washing with PBS, the samples were resuspended in PBS containing 200 µg/ml of RNAse A and 20 µg/ml of propidium iodide (PI) and incubated at room temperature for 30 min.

### Flow Cytometry Analysis

A minimum of 20,000 stained cells were acquired on a FACSCalibur Flow Cytometer (Becton Dickinson) equipped with a blue laser (488 nm). The Alexa 488 or the DyLight 488 fluorochromes were excited by the laser, and the emitted light collected (FL1 channel) via a 530/30 filter. The PI fluorescence was collected (FL3 channel) via a 670 long pass filter. No compensation was needed. The data were analyzed with the FCS Express 4 software.

Cell population was gated based on the Forward versus Side Scatter plot to exclude debris, and then single cells were gated by using a dot-plot or a density-plot showing the pulse width (FL3W) versus pulse area (FL3A) of the PI channel. After gating the cells by their DNA content, the Top2$\alpha$ or Top2$\beta$ intensity of fluorescence was evaluated. The Top2$\alpha$ or Top2$\beta$ intensity of fluorescence was displayed as a FL1 histogram and analyzed as median intensity of fluorescence (MIF). The complete gating strategy is depicted in supplemental Figure 1.

To compare salt- and heparin-extraction on HeLa cells, the Top2$\alpha$ intensity of fluorescence (FL1) was plotted versus FL3A. Based on the DNA content, three gates were performed representing the G1, S, and G2/M populations. As described previously (20), to compensate differences on DNA content, the Top2$\alpha$ MIF of gates corresponding to S and G2/M populations were multiplied by 0.75 and 0.5, respectively.

## Results

### Stabilized Top2cc Can Be Assessed by Flow Cytometry

In order to determine whether Top2$\alpha$ and Top2$\beta$ isoforms bound to chromatin of HL-60 cells can be recognized by flow cytometry following protein extraction with detergent and heparin, we immunolabeled either isoforms with specific antibodies that have been previously validated in immunofluorescence experiments (18,21,22). As shown in Figure 1A and 1B, both antibodies recognized their antigens providing sufficient signal intensities following extraction to differentiate them from their isotype controls and from samples that were not heparin-extracted.

As the poison ETO stabilize Top2$\alpha$cc and Top2$\beta$cc by increasing their half-life, we analyzed in HL-60 cells whether Top2cc stabilized by ETO can be detected following protein extraction with detergent and heparin. In Figure 1C and 1D is depicted the analysis of Top2$\alpha$cc and Top2$\beta$cc, respectively, following a 1 h treatment with ETO 10 μg/ml or vehicle (DMSO). As shown in Figure 1C (central dot plots), the intensity of fluorescence of Top2$\alpha$ was increased following treatment with ETO, showing different signal intensities in populations with different DNA content. This is in agreement with the cell cycle phase-dependent activity of Top2$\alpha$ previously reported (19,23). In the G1-phase cell population; however, a high variability in the signal intensities of Top2$\alpha$ following treatment with ETO was shown, probably as a result of protein turnover as cells exit mitosis and head toward the G1/S boundary. In the right panel of Figure 1C is represented the overlapped histograms of the Top2$\alpha$ intensities of fluorescence. On the other hand, the increased intensity of fluorescence of Top2$\beta$ following treatment with ETO shown in Figure 1D seems to be similar at different cell cycle stages. This is also in agreement with the constant activity of Top2$\beta$ reported previously throughout the interphase (23,24). The overlapped histograms of Top2$\beta$ intensities of fluorescence are shown in the right panel of Figure 1D.

To determine whether we could recognize Top2$\alpha$cc stabilized by other Top2 poisons, we incubated HL-60 cells with MTX and IDA (Figure 1E). Our results showed that Top2$\alpha$cc induced by either drug were also recognized using this approach.

Overall, these results suggest that this extraction method combined with immunolabeling detection can be used by flow cytometry to assess either isoforms of drug-stabilized Top2cc in HL-60 cells in a cell cycle context.

### Heparin-Extraction of Proteins Can Replace Salt-Extraction for Analyzing Drug-Stabilized Top2cc

Most proteins tightly bound to DNA are resistant to extraction with detergents and increasing salt conditions. This property has been widely used for chromatin fractionation experiments (25), and also for separating the unbound fraction of Top2 (19). However, high salt conditions disturb the chromatin organization in the nucleus by releasing large histone-free DNA loops that remain fixed to the nuclear matrix (26). In our experience, the classical extraction of Top2 with 350 mM NaCl for just 1 min was sufficient to induce the aggregation of non-adherent growing cells with the release of their chromatin loops (Supporting Information Fig. S2). This phenomenon was not seen by us on adherent growing cells. This observation limited the application of salt-based extraction methods of non-adherent cells for flow cytometry analysis.

Unfractionated heparin is the compound of highest negative charge density of the naturally occurring anionic polymers. This is due to its high content of negative charged sulfonate and carboxylate groups (27). Heparin-containing extraction buffers were utilized in the past for the gentle removal of proteins from DNA through competition with heparin (28).

In order to evaluate our method, we compared it with the previously validated DRT assay (19) in HeLa cells. As shown in Figure 2A and 2B, the extraction of Top2$\alpha$ fraction unbound or weakly bound to DNA was very similar using either approach. The analysis of data obtained by both extraction methods for the signals of ETO-stabilized Top2$\alpha$cc showed no significant differences throughout the cell cycle. Moreover, the number of cells showing Top2$\alpha$cc positive signals (Figure 2C and 2D) was very similar in both cases.

The histogram shown in Figure 2E depicts the overlapped intensities of fluorescence of Top2$\alpha$ following the extraction with different amounts of heparin for $1 \times 10^6$ HeLa cells per sample. As shown, less than 100U of unfractionated heparin lead to an incomplete removal of unbound Top2$\alpha$.

Together, these data demonstrate that the heparin-extraction of HeLa cells can be consistently used for the analysis of drug-stabilized Top2$\alpha$cc by flow cytometry, being as reliable as the salt-extraction based methods.

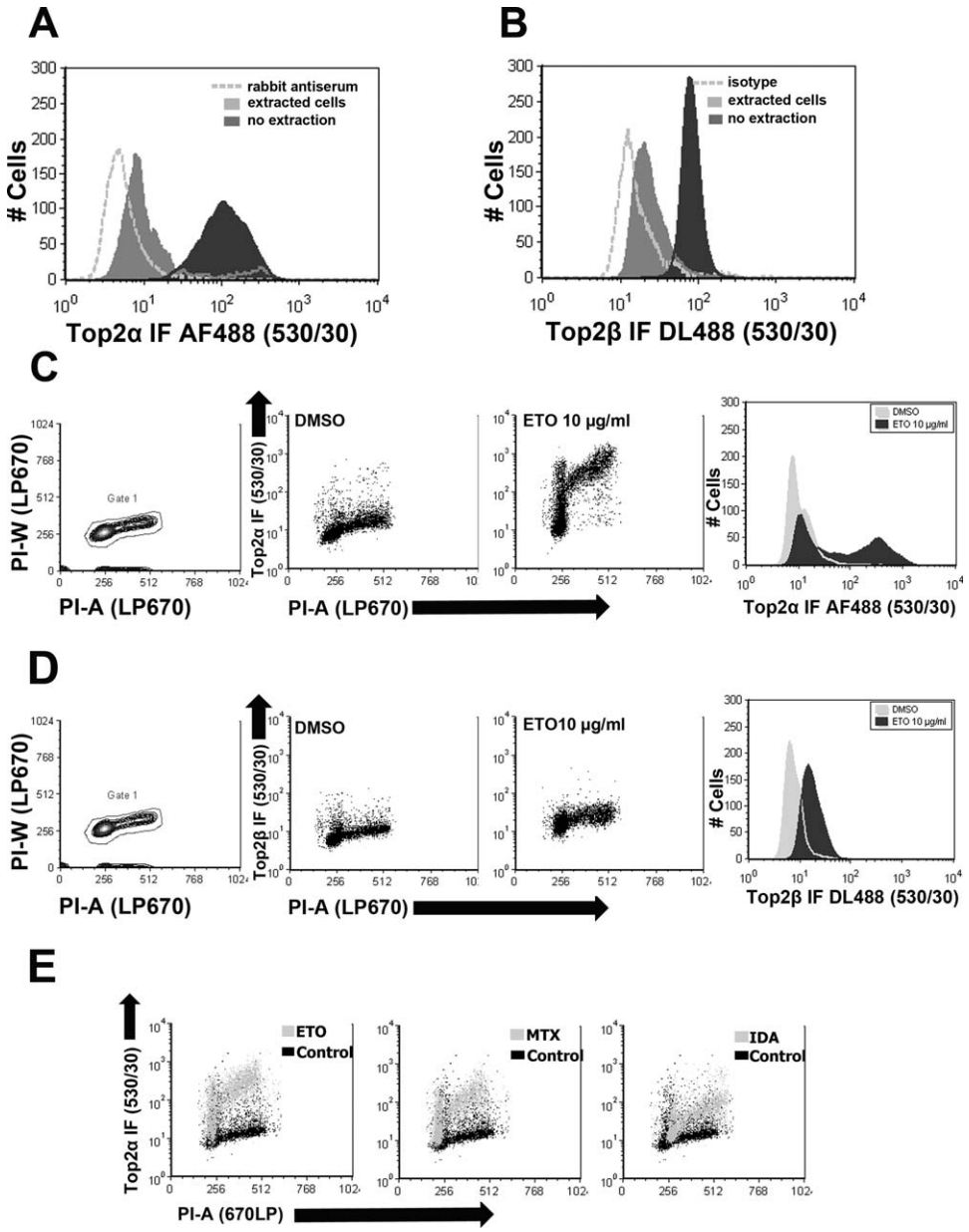


**Figure 1.** Top2cc can be analyzed by flow cytometry in HL-60 cell line. (**A**) Overlapped histograms showing the intensity of fluorescence (IF) of Top2$\alpha$ following or not heparin-extraction. In the negative control, the primary antibody was replaced by rabbit antiserum in a heparin-extracted sample. (**B**) Overlapping histograms showing the intensity of fluorescence of Top2$\beta$ following or not heparin extraction. The negative control was an isotype control that was also heparin-extracted. (**C**) DMSO 0.5% or ETO 10 μg/ml treated cells were heparin-extracted and following Top2$\alpha$ immunolabeling, they were counterstained with PI. (left) A density blot of FL-3A vs. FL-3W was generated to gate the cell population. (center) Dot plots analysis of the gated population shows the cell cycle phase-dependent increase of the intensity of fluorescence of Top2$\alpha$ after treatment with ETO compared to DMSO. (right) Overlapped histograms showing the intensities of fluorescence (IF) of Top2$\alpha$ are represented. (**D**) The analysis shown in (C) was also performed for Top2$\beta$. A density plot was performed to gate the cell population (left) and a dot plot analysis of the gated population was performed (center). In this case, a cell cycle-independent increase of the intensity of fluorescence of Top2$\beta$ was shown following treatment with ETO 10 μg/ml. (right) The overlapped histogram of the intensity of fluorescence of Top2$\beta$ is also shown. (**E**) Representative overlapped dot blots analysis showing the increased intensity of fluorescence of Top2$\alpha$ following a 1 h incubation with ETO 10 μg/ml (left), MTX 1 μM (center), or IDA 0.5 μg/ml (right) compared to an untreated control.

### Poisoning Different Isoforms of Top2 and Different Cell Types Can Be Assayed for Cleavage Complex Formation by Flow Cytometry

In order to determine whether the poisoning of different isoforms of Top2 by ETO is dose-dependent, we incubated K-562 cell line by 1 h with different doses of the drug. As shown in Figure 3A, ETO-stabilized Top2αcc was detected from 2 to 150 μg/ml, reaching almost a plateau at 10 μg/ml. By contrast, Figure 3B shows that poisoning

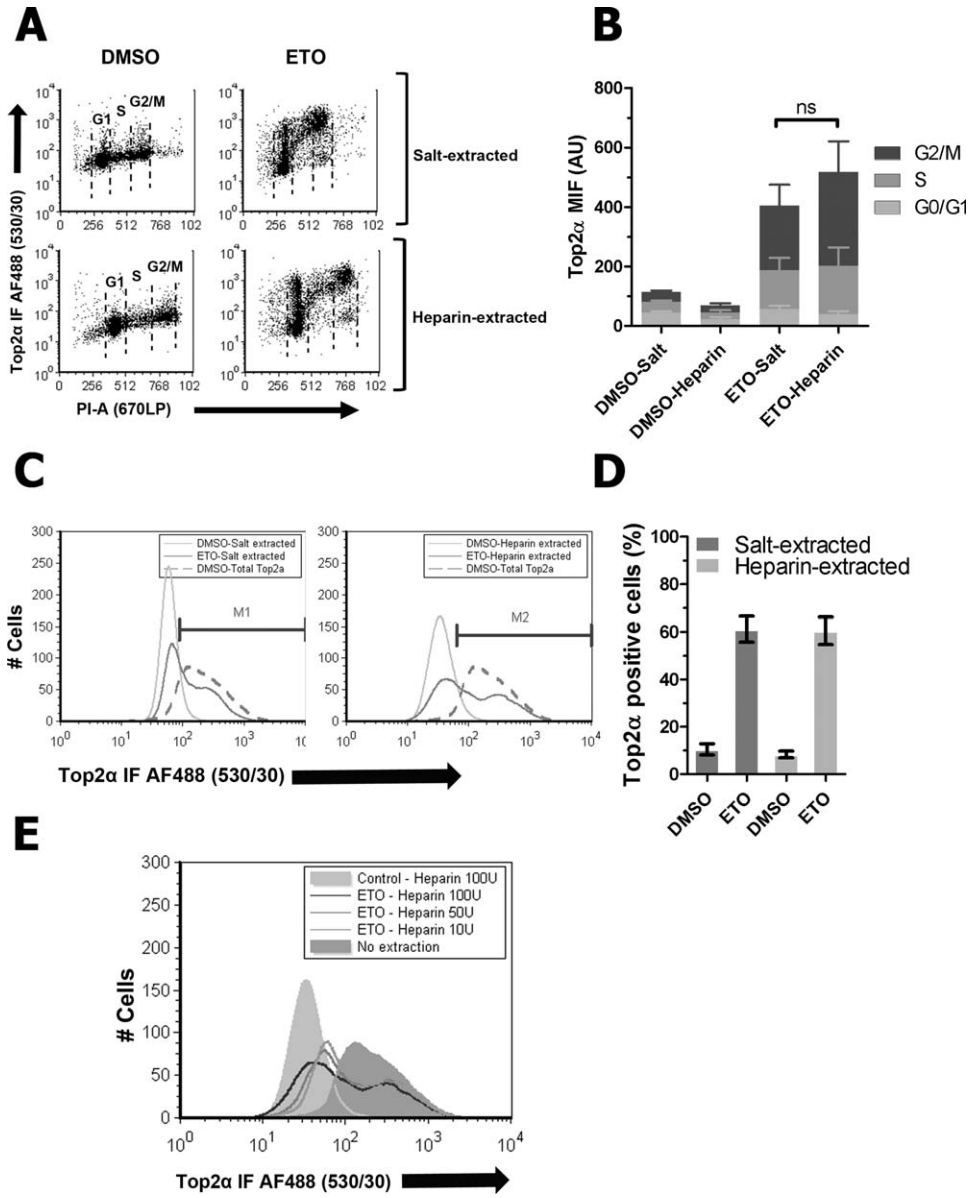


**Figure 2.** Comparison of heparin- and salt-extraction of Top2 in adherent cells by flow cytometry. (**A**) Dot plot analysis of HeLa cells treated with ETO 10 μg/ml or vehicle (DMSO 0.5%) and extracted in the presence of heparin (100U/ml) by 5 min or salt (NaCl 350 mM) by 2 min. The discrimination of the different cell cycle phases was performed by counterstaining with PI. (**B**) The median intensity of fluorescence (MIF) of Top2α was calculated ($n = 3$) for each cell cycle phase as described in Materials and Methods section. ns = no significant statistical differences (Student *t*-test). Error bars correspond to standard deviation (SD) of the mean. (**C**) Representative overlapped histograms showing the intensities of fluorescence of Top2α after salt (left) and heparin (right) extraction. In both cases, the dashed lines represent a control sample that was not extracted (total Top2α content). Markers, M1 and M2, were performed to calculate the percentage of positives cells for Top2α ($n = 3$), which was quantified and represented in (**D**). These markers were drawn to contain >90% of the control not extracted cell population (dashed lines). (**E**) ETO-treated cells were extracted with different doses of unfractionated heparin.

Top2β was more evident with increasing doses of ETO. The finding of a dose-dependent effect of ETO in poisoning different Top2 isoforms are in line with previous reports (29).

To test whether this method can be used in different cell types, different cell lines and cultures were assayed (Figure 3C). As shown, this method allowed us to detect ETO-stabilized Top2αcc in both chronic myeloid leukemia and neuroblastoma cell lines as well as stimulated PBL cultures. This, in addition to the results obtained with the promyeloblast HL-60 cell line (Figure 1), the adenocarcinoma HeLa cells (Figure 2), and the normal human fibroblast PTP cell line (data not shown) argues that this assay is suitable for most cell suspension and adherent growing cells.

### Removal of Drug-Stabilized Top2cc Can Be Monitored by Flow Cytometry

We next examined whether the removal of Top2cc can be analyzed by this method. Thus, we incubated the HL-60

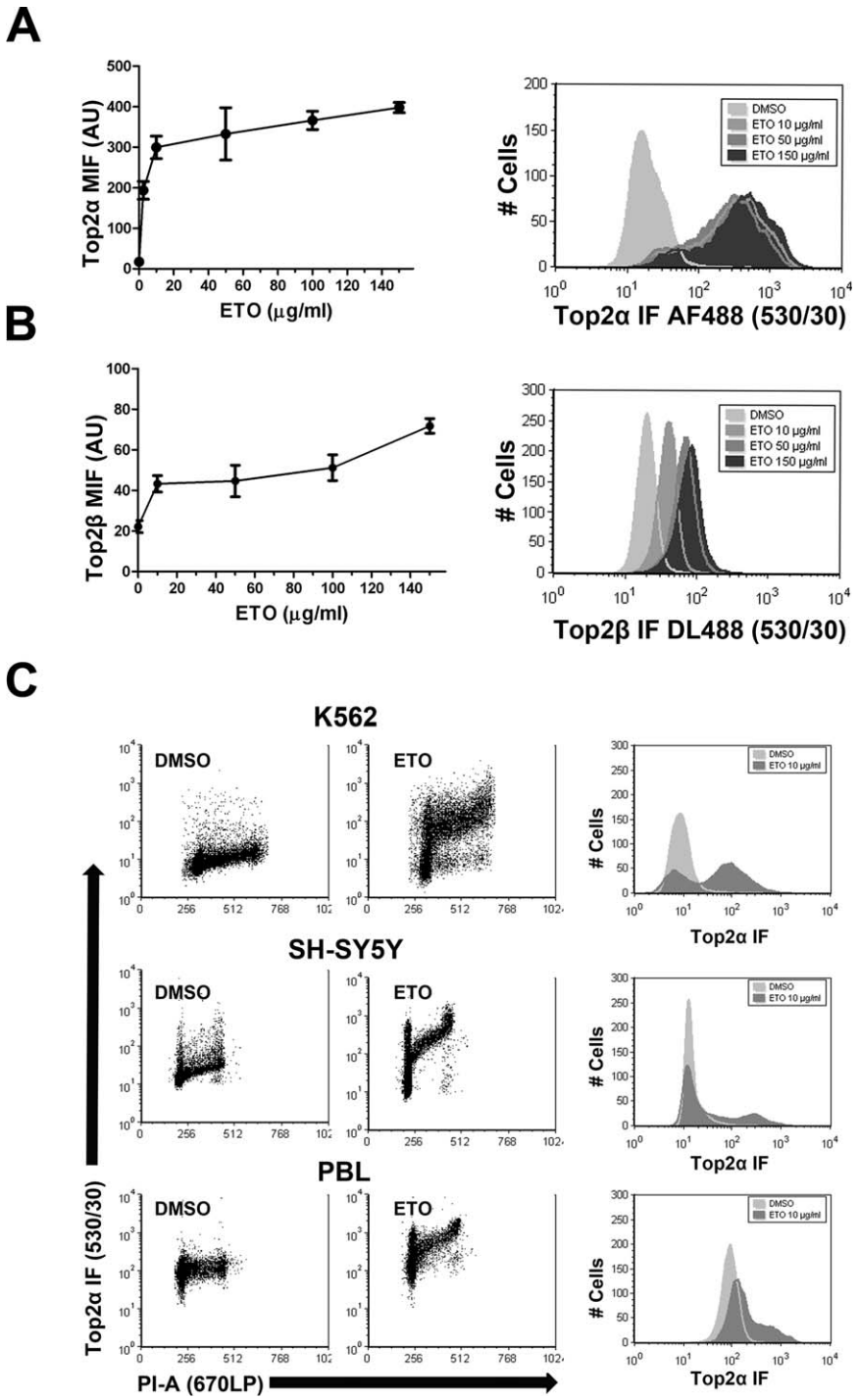


**Figure 3.** Poisoning different isoforms of Top2 and different cell types can be analyzed by flow cytometry. (**A**) Median intensity of fluorescence (MIF) of Top2$\alpha$ (left) following increasing dose of ETO in K-562 cell line ($n = 4$). A representative experiment is depicted in the overlapped histogram (right). (**B**) Median intensity of fluorescence (MIF) of Top2$\beta$ (left) following increasing dose of ETO ($n = 3$). A representative experiment is also shown in the overlapped histogram (right). AU = arbitrary units. (**C**) Dot plot analysis (left) of Top2$\alpha$ poisoning following a 1 h treatment with ETO on K-562 and SH-SY5Y cell lines and stimulated peripheral blood lymphocytes (PBL). (right) The overlapped histogram of the intensities of fluorescence for Top2$\alpha$ is also shown.

cells with ETO by 1 h and harvested or allowed them to recover by different lapses of time. As a proteasome-dependent pathway was previously shown to be involved in the removal of Top2cc (8,30), we performed our experiences in the presence or not of the 26S proteasome inhibitor Bortezomib (2.5 μM, Btz).

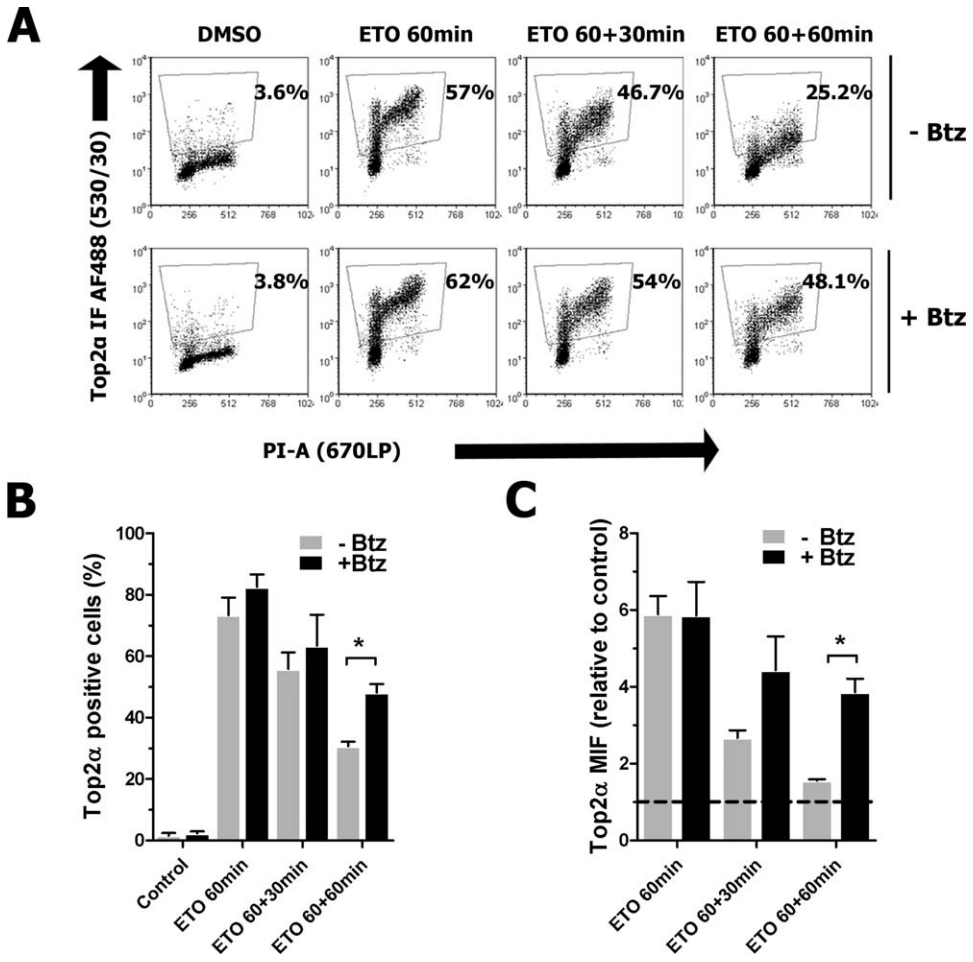


**Figure 4**. Drug-stabilized Top2αcc removal can be followed up by flow cytometry. HL-60 cells pre-incubated or not by 2 h with the 26S proteasome inhibitor Bortezomib (Btz, 2.5 μM) were treated with ETO 10 μg/ml by 1 h and harvested or allowed to recover in ETO-free complete media by different periods of times (30 and 60 min). (**A**) Dot plot analysis of Top2αcc formation and removal following treatment with ETO alone (top) or in the presence of Btz (bottom). A gate was created to calculate the percentage of cells in it. (**B**) The quantification of Top2α positive cells from three to four independent experiments is shown. (**C**) The median intensities of Top2α signals are represented. (*) Denotes statistically significant differences ($P < 0.05$; Student's *t*-test). Error bars correspond to the standard error of the mean (SEM).

In the upper panels of Figure 4A, it is shown dot plots of a representative experiment for the kinetics of Top2αcc formation and removal following treatments with ETO. A gate was performed for the analysis of the number of Top2α positive cells. In the lower panels, it is shown the same analysis but on cells pre-incubated by 2 h with Btz, which was present during all the culture time. As detailed in Figure 4B, the percentage of cells showing Top2α signals following treatment with ETO was similar. However, after 1 h of recovery, the pre-treatment with Btz caused a significantly higher percentage of cells retaining Top2α. The analysis of the median intensity of fluorescence (Figure 4C) showed comparable results. Cells exposed to ETO and allowed to recovery by 1 h showed a reduction from 5.9 to 1.56 fold. As expected, in the presence of Btz the intensity of fluorescence of Top2α after 1 h of recovery was reduced from 5.85 to 3.86 fold. These results are in line with the notion of an involvement of the 26S proteasome in the removal of Top2α isoform.

The same analysis was performed on ETO-induced Top2βcc (Figure 5). A representative experiment is exposed in Figure 5A. As depicted in Figure 5B, the presence of Btz had a more profound impact on the kinetics of Top2βcc formation and removal. The percentage of cells showing Top2β signals was significantly increased in the presence of Btz (∼58%), probably reflecting an accumulation of ETO-induced Top2βcc during the exposure time. In addition, Top2βcc showed a more rapid kinetics of removal than Top2αcc. At 30 min of recovery, the percentage of cells retaining Top2β positive signals was significantly increased in cells pre-treated with Btz. As shown in Figure 5C, the intensity of fluorescence in the absence of Btz was reduced from 2.5 to 1.28 fold, while the presence of Btz caused a reduction from 3.5 to 2.1 fold.

Together, these results argue that this assay is appropriate to follow the kinetics of Top2cc removal.

## Discussion

Early biochemical studies by anion-exchange chromatography of partially purified human Top2 identified different

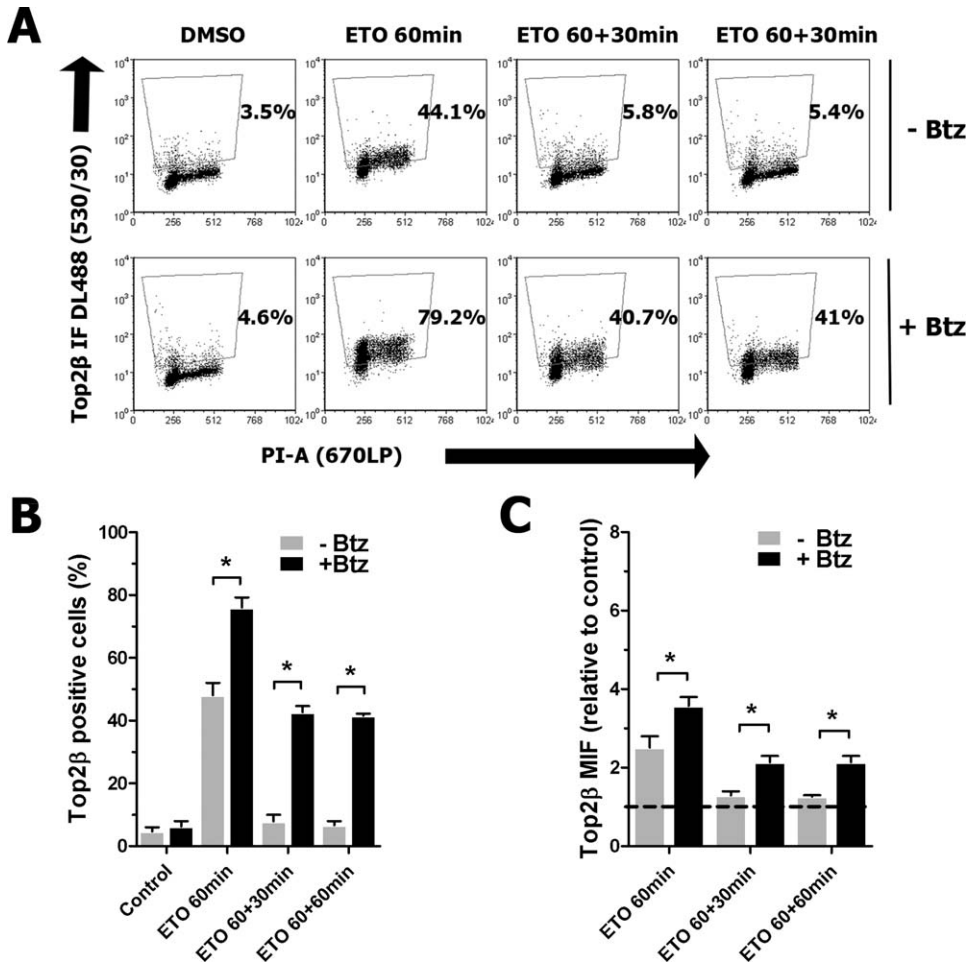


**Figure 5.** Drug-stabilized Top2$\beta$cc removal can be tracked by flow cytometry. HL-60 cells were treated as in Figure 4. (**A**) Dot plot analysis of Top2$\beta$cc formation and removal following treatment with ETO alone (top) or in the presence of Btz (bottom). A gate was performed to calculate the percentage of cells in it. (**B**) The quantification of Top2$\beta$ positive cells from three to four independent experiments is shown. (**C**) The median intensities of fluorescence of Top2$\beta$ signals are represented. (*) Denotes statistically significant differences ($P < 0.05$, Student's *t*-test). Error bars correspond to the standard error of the mean (SEM).

fractions of catalytically active enzyme. Among them, the most resistant fraction of Top2$\alpha$ that retained catalytic activity was resolved at 300–400 mM NaCl, while Top2$\beta$ was at 230 mM NaCl (31). This observation outlined several salt extraction methods for revealing Top2cc after removing the unbound or weakly-bound enzyme from cells (19,23). Other strategy to reveal covalent protein-DNA adducts was the generation of specific antibodies. This was successfully performed for Top1-DNA complexes (32), but not for Top2-DNA. Although this method seems to be more specific and sensitive, it is limited by the lack of commercially available antibodies.

We have developed here a flow cytometry-based assay that utilizes anti-Top2$\alpha$ or anti-Top2$\beta$ commercially available antibodies to detect drug-stabilized Top2 cleavage complexes. The heparin-extraction of weakly-bound Top2 by competition with DNA before sample fixation, allowed us to discriminate the tightly associated fraction of Top2 which remains covalently linked to DNA. Heparin extraction also permits the simultaneous follow up of Top2cc removal and DNA damage development through the induction of $\gamma$H2AX (data not shown). However, the removal of other components of the DDR was not assessed and may represent the main disadvantage of this method. The use of unfractionated heparin was also valuable to circumvent the aggregation of non-adherent growing cells that release their DNA loops following increased salt conditions; thus, making this method suitable for most cell types.

We have determined that this assay can recognize increased Top2$\alpha$ or Top2$\beta$ signals after being trapped on DNA by exposure to Top2 poisons. Moreover, our data show that Top2cc stabilized by different interfacial Top2 poisons such as IDA, ETO and MTX can be revealed. Both IDA and MTX bind DNA by intercalation (33,34). This suggests that the method is suitable to assay drugs that poison Top2 independently of their property of intercalating DNA.

The significant differences in both the percentage of cells showing Top2 positive staining and the intensity of the signals after poisoning Top2 allows a reliable quantification of Top2cc and tracking its removal. Moreover, the combined use of a

DNA intercalating dye such as propidium iodide allows the detection of Top2cc contextualized on the different phases of the cell cycle.

The low processing time and versatility in the kind of samples to be analyzed are the main advantages of this method. These properties combined with the use of flow cytometry favor a high-throughput analysis of drug-stabilized Top2cc, which would be useful in the field of drug-discovery. The screening for drugs that poison a specific-isoform of Top2 would also gain from this assay. The fact that there is no restriction in the number of cells to be analyzed bypasses the most limiting aspect of the microscopy-based methods, leading to a more consistent statistical analysis.

Drug-stabilized Top2cc are removed by a complementary network of hydrolytic (17,18), nucleolytic (16,35), and proteolytic (8,30) pathways through diverse cellular processes. Genetic knock-down of proteins involved in Top2cc removal pathways can lead to increased susceptibility to Top2 poisons (18,36). Proteosomal inhibition-mediated stabilization of Top2 was also shown to revert the resistant phenotype to Top2 poisons on solid and hematological tumors (37,38). On the other hand, overexpression of proteins that remove Top2cc can result in resistance to Top2 poisons (39). Therefore, this method may help to understand better the interrelationship between pathways involved in the removal of drug-stabilized Top2cc. Moreover, as previously suggested for the analysis Top2 expression in breast cancers (40), this procedure may be useful to identify subsets of tumor cells resistant to drug-stabilized Top2cc formation, which may correlate with the progression of the disease. Clinical samples may also be assayed for tracking the removal of Top2cc which can shed light on whether a Top2 poison-based therapy may be beneficious or not, even when the genetic background of the cell population being studied is unknown.

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## Literature Cited

1. Wang JC. DNA topoisomerases. Ann Rev Biochem 1996;65:635–692.
2. Warburton PE, Earnshaw WC. Untangling the role of DNA topoisomerase II in mitotic chromosome structure and function. BioEssays 1997;19:97–99.
3. Heck MM, Hittelman WN, Earnshaw WC. Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. Proc Natl Acad Sci USA 1988;85: 1086–1090.
4. Capranico G, Tinelli S, Austin CA, Fisher ML, Zunino F. Different patterns of gene expression of topoisomerase II isoforms in differentiated tissues during murine development. Biochim Biophys Acta 1992;1132:43–48.
5. Wang JC. Cellular roles of DNA topoisomerases: A molecular perspective. Nat Rev Mol Cell Biol 2002;3:430–440.
6. Grue P, Grasser A, Sehested M, Jensen PB, Uhse A, Straub T, Ness W, Boege F. Essential mitotic functions of DNA topoisomerase IIalpha are not adopted by topoisomerase IIbeta in human H69 cells. J Biol Chem 1998;273:33660–33666.
7. Ju BG, Lunyak VV, Perissi V, Garcia-Bassets I, Rose DW, Glass CK, Rosenfeld MG. A topoisomerase IIbeta-mediated dsDNA break required for regulated transcription. Science 2006;312:1798–1802.
8. Mao Y, Desai SD, Ting CY, Hwang J, Liu LF. 26 S proteasome-mediated degradation of topoisomerase II cleavable complexes. J Biol Chem 2001;276:40652–40658.
9. Pommier Y, Leo E, Zhang H, Marchand C. DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. Chem Biol 2010;17:421–433.
10. Bandele OJ, Osheroff N. Bioflavonoids as poisons of human topoisomerase II alpha and II beta. Biochemistry 2007;46:6097–6108.
11. Kingma PS, Greider CA, Osheroff N. Spontaneous DNA lesions poison human topoisomerase IIalpha and stimulate cleavage proximal to leukemic 11q23 chromosomal breakpoints. Biochemistry 1997;36:5934–5939.
12. Pendleton M, Lindsey RH Jr., Felix CA, Grimwade D, Osheroff N. Topoisomerase II and leukemia. Ann N Y Acad Sci 2014;1310:98–110.
13. Nitiss JL. DNA topoisomerase II and its growing repertoire of biological functions. Nat Rev Cancer 2009;9:327–337.
14. Bandele OJ, Osheroff N. The efficacy of topoisomerase II-targeted anticancer agents reflects the persistence of drug-induced cleavage complexes in cells. Biochemistry 2008;47:11900–11908.
15. Nitiss JL, Beck WT. Antitopoisomerase drug action and resistance. Eur J Cancer 1996;32A:958–966.
16. Hartsuiker E, Neale MJ, Carr AM. Distinct requirements for the Rad32(Mre11) nuclease and Ctp1(CtIP) in the removal of covalently bound topoisomerase I and II from DNA. Mol Cell 2009;33:117–123.
17. Cortes Ledesma F, El Khamisy SF, Zuma MC, Osborn K, Caldecott KW. A human 5′-tyrosyl DNA phosphodiesterase that repairs topoisomerase-mediated DNA damage. Nature 2009;461:674–678.
18. Borda MA, Palmitelli M, Verón G, González-Cid M, de Campos-Nebel M. Tyrosyl-DNA-Phosphodiesterase I (TDP1) participates in the removal and repair of stabilized-Top2α cleavage complexes in human cells. Mutat Res 2015;781:37–48.
19. Agostinho M, Rino J, Braga J, Ferreira F, Steffensen S, Ferreira J. Human topoisomerase IIalpha: Targeting to subchromosomal sites of activity during interphase and mitosis. Mol Biol Cell 2004;15:2388–2400.
20. Huang X, Okafuji M, Traganos F, Luther E, Holden E, Darzynkiewicz Z. Assessment of histone H2AX phosphorylation induced by DNA topoisomerase I and II inhibitors topotecan and mitoxantrone and by the DNA cross-linking agent cisplatin. Cytometry, Part A 2004;58:99–110.
21. de Campos-Nebel M, Larripa I, Gonzalez-Cid M. Topoisomerase II-mediated DNA damage is differently repaired during the cell cycle by non-homologous end joining and homologous recombination. PloS One 2010;5:e12541.
22. Li Y, Hao H, Tzatzalos E, Lin RK, Doh S, Liu LF, Lyu YL, Cai L. Topoisomerase IIbeta is required for proper retinal development and survival of postmitotic cells. Biol Open 2014;3:172–184.
23. Meyer KN, Kjeldsen E, Straub T, Knudsen BR, Hickson ID, Kikuchi A, Kreipe H, Boege F. Cell cycle-coupled relocation of types I and II topoisomerases and modulation of catalytic enzyme activities. J Cell Biol 1997;136:775–788.
24. Austin CA, Marsh KL. Eukaryotic DNA topoisomerase II beta. BioEssays 1998;20: 215–226.
25. Mirzoeva OK, Petrini JH. DNA damage-dependent nuclear dynamics of the Mre11 complex. Mol Cell Biol 2001;21:281–288.
26. Iarovaia OV, Akopov SB, Nikolaev LG, Sverdlov ED, Razin SV. Induction of transcription within chromosomal DNA loops flanked by MAR elements causes an association of loop DNA with the nuclear matrix. Nucl Acids Res 2005;33:4157–4163.
27. Capila I, Linhardt RJ. Heparin-protein interactions. Angew Chem Int Ed Engl 2002; 41:391–412.
28. Adolphs KW, Cheng SM, Paulson JR, Laemmli UK. Isolation of a protein scaffold from mitotic HeLa cell chromosomes. Proc Natl Acad Sci USA 1977;74:4937–4941.
29. Tammaro M, Barr P, Ricci B, Yan H. Replication-dependent and transcription-dependent mechanisms of DNA double-strand break induction by the topoisomerase 2-targeting drug etoposide. PloS One 2013;8:e79202.
30. Zhang A, Lyu YL, Lin CP, Zhou N, Azarova AM, Wood LM, Liu LF. A protease pathway for the repair of topoisomerase II-DNA covalent complexes. J Biol Chem 2006; 281:35997–36003.
31. Boege F, Kjeldsen E, Gieseler F, Alsner J, Biersack H. A drug-resistant variant of topoisomerase II alpha in human HL-60 cells exhibits alterations in catalytic pH optimum, DNA binding and sub-nuclear distribution. Eur J Biochem 1993;218:575–584.
32. Patel AG, Flatten KS, Peterson KL, Beito TG, Schneider PA, Perkins AL, Harki DA, Kaufmann SH. Immunodetection of human topoisomerase I-DNA covalent complexes. Nucl Acids Res 2016;44:2816–2826.
33. Kapuscinski J, Darzynkiewicz Z. Interactions of antitumor agents Ametantrone and Mitoxantrone (Novatrone) with double-stranded DNA. Biochem Pharmacol 1985; 34:4203–4213.
34. Belloc F, Lacombe F, Dumain P, Lopez F, Bernard P, Boisseau MR, Reifers J. Intercalation of anthracyclines into living cell DNA analyzed by flow cytometry. Cytometry 1992;13:880–885.
35. Tammaro M, Liao S, Beeharry N, Yan H. DNA double-strand breaks with 5' adducts are efficiently channeled to the DNA2-mediated resection pathway. Nucl Acids Res 2016;44:221–231.
36. Nakamura K, Kogame T, Oshiumi H, Shinohara A, Sumitomo Y, Agama K, Pommier Y, Tsutsui KM, Tsutsui K, Hartsuiker E, et al. Collaborative action of Brca1 and CtIP in elimination of covalent modifications from double-strand breaks to facilitate subsequent break repair. PLoS Genet 2010;6:e1000828.
37. Ogiso Y, Tomida A, Lei S, Omura S, Tsuruo T. Proteasome inhibition circumvents solid tumor resistance to topoisomerase II-directed drugs. Cancer Res 2000;60:2429–2434.
38. Congdon LM, Pourpak A, Escalante AM, Dorr RT, Landowski TH. Proteasomal inhibition stabilizes topoisomerase IIalpha protein and reverses resistance to the topoisomerase II poison ethonafide (AMP-53, 6-ethoxyazonafide). Biochem Pharmacol 2008;75:883–890.
39. Barthelmes HU, Habermeyer M, Christensen MO, Mielke C, Interthal H, Pouliot JJ, Boege F, Marko D. TDP1 overexpression in human cells counteracts DNA damage mediated by topoisomerases I and II. J Biol Chem 2004;279:55618–55625.
40. Epstein RJ, Smith PJ, Watson JV, Waters C, Bleehen NM. Oestrogen potentiates topoisomerase-II-mediated cytotoxicity in an activated subpopulation of human breast cancer cells: Implications for cytotoxic drug resistance in solid tumours. Int J Cancer 1989;44:501–505.