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Pharmacologic topoisomerase-I inhibition causes DNA damage and mortality in activated CD4$^+$ T cells

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Abstract

Topoisomerase-I is required for DNA replication. It acts by preventing torsional stress caused by DNA winding during replication fork progression. Topoisomerase-I inhibitors are widely used in many cancer therapies, in light of their anti-proliferative activity. However, their use as chemotherapeutics is associated with significant toxicity due to the off-target effects on healthy cells. We analyzed the dose-time-toxicity profile of a clinically employed topoisomerase-I inhibitor, *i.e.* topotecan, on primary CD4$^+$T cells. This cell type was chosen to model a typical *in-vivo* interaction, due to the wide use of topotecan in the treatment of T-cell lymphomas. Our results show that a clinically achievable concentration of topotecan can induce toxic effects in healthy CD4$^+$ T cells as early as 7 hours of the *in vitro* treatment. Toxicity of the drug was markedly increased by prolonging the post-treatment follow-up, but not by increasing concentrations, suggesting that clinical doses of topotecan can induce cell death and DNA damage in non-cancerous activated CD4$^+$ T lymphocytes.

Keywords

Topoisomerase-I, topotecan, γH2AX, CD4$^+$ T cells

Introduction

DNA topoisomerases are ubiquitously expressed in eukaryotic cells, with six different types being expressed in human cells. All DNA topoisomerases are involved in the regulation of DNA topological stress caused by DNA strand separation in the processes of DNA replication and transcription (Watson & Crick, 1953; Wang, 2002).

Topoisomerases act on a broad range of metabolic processes including both DNA and RNA substrates in nuclear and mitochondrial genomes (Pommier et al., 2016). The main function of topoisomerases is the introduction of transient DNA breaks by transesterification (Wang, 2002; Pommier et al., 2010; Chen et al., 2013).

Given their role during DNA replication and transcription, topoisomerases represent important drug targets in bacterial and eukaryotic cells and are therapeutic targets for treating various types of cancers, as well as immune and neurological disorders (Pommier et al., 2010; Nitiss, 2009).

Among the drugs targeting topoisomerases, topotecan is the first clinically approved compound to inhibit the action of type I topoisomerase (topoisomerase-I) (Takimoto & Arbuck, 1996; Jonnson et al., 1997). Topoisomerase-I is a nuclear member of the topoisomerase family, which is specifically involved in the unwinding of supercoiled DNA, formed during replication, by binding to it and introducing a cut in one of the DNA strands.

Through its action on topoisomerase-I, topotecan was shown to exert a cytotoxic effect *in vitro* on a wide range of different cell types (Burris et al., 1992; Uckun et al., 1995), as well as *in vivo* in mice bearing human tumor xenografts (Friedman et al., 1994; Houghton et al., 1995). Also, the clinical activity of topotecan was shown in cases of ovarian carcinoma (Gore et al., 1996), non-small-cell lung cancer (Perez-Soler et al., 1996) and leukemia (Kantarjian et al., 1993; Rowinsky et al., 1994).

While potential side effects of topotecan on myeloid cells and thrombocytes have been reported (Andreopoulou et al., 2010), its effects on human lymphocytes is less known. In particular, we hypothesized that activation of T-lymphocytes might render them susceptible to topotecan due to the increased metabolic activity. In order to assess potentially deleterious effects of topoisomerase-I inhibition in healthy human cells, primary $CD4^+$ T cells were activated and treated with different concentrations of topotecan and analyzed for cell viability and DNA damage markers at different time points post treatment.

Materials and methods

Cell isolation and cell culture

Primary $CD4^+$ T cells were purified from whole blood from healthy donors with RosetteSep™ Human $CD4^+$ T Cell Enrichment Cocktail (Stemcell Technologies). Cells were activated with Dynabeads Human T-Activator CD3/CD28 (ThermoFisher) and 5 ng/mL IL-2 for 48hr and kept in RPMI medium supplemented with 10 % FBS and 0,1 mg/mL Primocin (Marini et al., 2015).

Drug treatment

Primary $CD4^+$ T cells were treated with 0,25; 0,5 and 25μM topotecan hydrochloride hydrate (Sigma-Aldrich) for either 3 or 7 hours and collected for cell viability assay or fixed for immunofluorescence (IF) staining.

The different drug concentrations were chosen to include a range of different concentrations used in previous studies on different cell lines originating from different cancer types (Danks et al., 1988; Botling et al., 1994; Jonsson et al., 1997; Hassan et al., 2001) and, on the lower end, a potentially achievable level of topotecan in plasma (Andreopoulou et al., 2010).

For follow-up on long term effects of topotecan, cells were washed with PBS after a 7-hour incubation with the aforementioned concentrations of the drug, kept in culture for another 72 hours and tested for cell viability.

Cell viability assay

Cell viability was determined by performing live cell staining and flow cytometry. $2*10^5$ cells were collected for each analyzed time point. As a negative control to gate dead cells, the same amount of cells was treated for 10 minutes at room temperature with 100% ethanol. Cells were washed in PBS and stained for 30 minutes on ice with the Fixable Viability Dye eFluor 450 (eBioscience). After the staining the cells were washed in PBS and fixed for 10 minutes in 4 % PFA/PBS. Flow cytometry was performed using a BD FACSVerse flow cytometer and flow cytometry data was analyzed with the BD FACSuite software (BD Biosciences; Franklin Lakes, New Jersey, USA).

Immunofluorescence staining

Used antibodies:

Used primary antibodies: rabbit polyclonal Lamin B1 16048 (Abcam) (stains the nuclear envelope) or mouse monoclonal mAb 414 24609 (Abcam) (stains phenylalanine-glycine repeats of nuclear pore complex proteins) to mark the nuclear rim, and mouse monoclonal Anti-phospho-Histone H2A.X (Ser139) 05-636 (Milipore) (stains DNA double strand breaks) to quantify potentially occurred DNA damage.

Cell preparation:

The cells were seeded and fixed with 4 % PFA/PBS on glass cover slips which were previously coated with 0,5 mg/mL PEI (Polyethyleneimine).

The cells were washed with PBS and premeabilized with 0,5 % Triton in 0,1% PBS-Tween for 10 minutes at room temperature. After washing and 30 minutes blocking in 4 % BSA/PBS the cells were incubated with primary antibodies diluted in a ratio of 1:500 in 1 % BSA/PBS over night at 4°C. After incubation with primary antibodies, cells were washed and incubated with secondary Alexa Fluor antibodies (ThermoFisher) diluted in a ratio of 1:1000 in 1 % BSA/PBS for 1 hr at room temperature. After washing, the cells were stained with Hoechst and mounted on glass slides with moviol solution (adapted from Marini et al., 2015).

The IF staining of DNA double strand marker γH2AX was was performed on cells treated with topotecan for 7 hours and 72 hours post drug removal. For this analysis we selected the lowest concentration employed in this study (0,25μM) as it is within the range of previously described clinically achievable concentrations.

Microscopy and image analysis

Confocal microscopy was performed on the confocal laser scanning microscope Leica TCS SP8.

Images were analyzed in ImageJ Fiji image processing package and Volocity imaging software (Perkin Elmer).

Results and Discussion

Primary $CD4^+$ T cells were treated with different concentrations of topotecan hydrochloride hydrate (Sigma-Aldrich) (0,25; 0,5 and 25μM).

In order to test the kinetic effects of the drug, activated primary $CD4^+$ T cells were tested for cell viability at different time points post drug treatment and drug release.

As early as 3 hours post-treatment an initial increase in cell mortality, with all the used concentrations of topotecan, could be observed (Figure 1; Table 1).

This effect was amplified at 7 hours post-treatment, with the cells treated with topotecan displaying an average increase of 11,21 % in cell death (Figure 2; Table 1 and 2).

To further test the longer-term consequences of topotecan treatment, the drug was washed from the cells after 7 hours of treatment, and the cells were kept for an additional 72 hours in culture and subsequently tested for their viability. Strikingly, despite the washing step, 72 hours post drug removal, all cultures previously treated with topotecan display

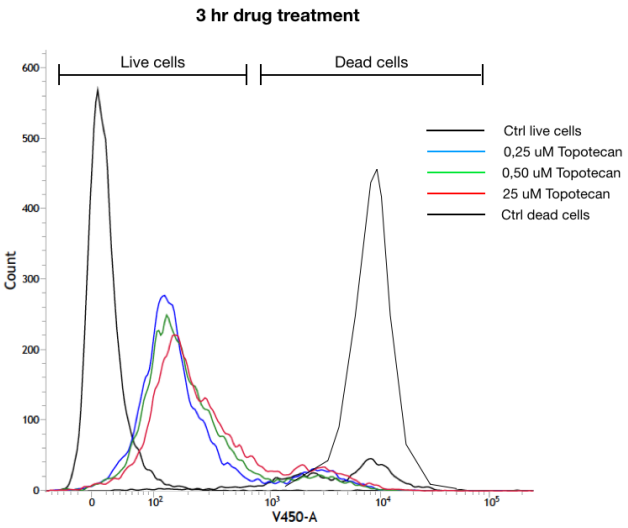


Figure 1. FACS profiles of primary $CD4^+$ T cells 3 hours post topotecan treatment. Primary $CD4^+$ T cells were activated with CD3/28 activating beads and IL-2 for 48 hr. Upon activation cells were treated with different concentrations of topotecan (Color code on the right shows control samples and different concentrations of administered drug) for 3 hr and subsequently analyzed by FACS to test for cell viability/mortality.

Table 1. Total cell count 3 hr post topotecan treatment

	Live cells (%)	Dead cells (%)
Ctrl live cells	88.87	11.13
0,25 uM topotecan	84.03	15.97
0,50 uM topotecan	82.38	17.62
25 uM topotecan	72.54	27.46
Ctrl dead cells	7.91	92.09

Table 2. Total cell count 7 hr post topotecan treatment

	Live cells (%)	Dead cells (%)
Ctrl live cells	75,68	24,32
0,25 uM topotecan	71,77	28,23
0,50 uM topotecan	72,54	27,46
25 uM topotecan	61,01	38,99
Ctrl dead cells	1,38	98,62

complete, or almost complete, cell death (Figure 3; Table 3).

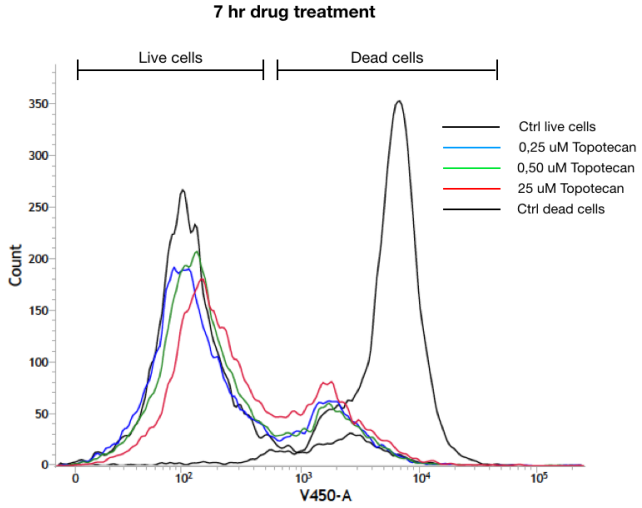


Figure 2. FACS profiles of primary CD4$^+$ T cells 7 hours post topotecan treatment. Primary CD4$^+$ T cells were activated with CD3/28 activating beads and IL-2 for 48 hr. Upon activation cells were treated with different concentrations of topotecan (Color code on the right shows control samples and different concentrations of administered drug) for 7 hr and subsequently analyzed by FACS to test for cell viability/mortality

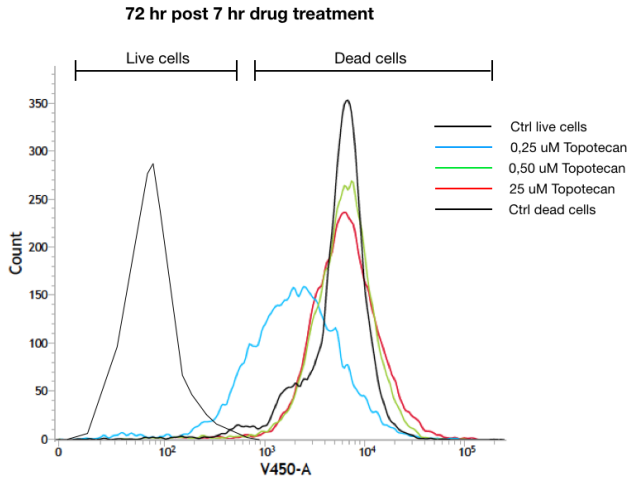


Figure 3. FACS profiles of primary CD4$^+$ T cells 72 hours post 7 hours topotecan treatment. Primary CD4$^+$ T cells were activated with CD3/28 activating beads and IL-2 for 48 hr. Upon activation cells were treated with different concentrations of topotecan (Color code on the right shows control samples and different concentrations of administered drug) for 7 hr and 72 hr post drug release analyzed by FACS to test for cell viability/mortality

Of note, a similar effect of topotecan has previously been described in lymphoma cell lines (Hassan et al., 2001).

Table 3. Total cell count 72 hr post 7 hr drug treatment

	Live cells (%)	Dead cells (%)
Ctrl live cells	81,23	18,77
0,25 uM topotecan	5,49	94,51
0,50 uM topotecan	0,95	99,05
25 uM topotecan	0,52	99,48
Ctrl dead cells	0,21	99,79

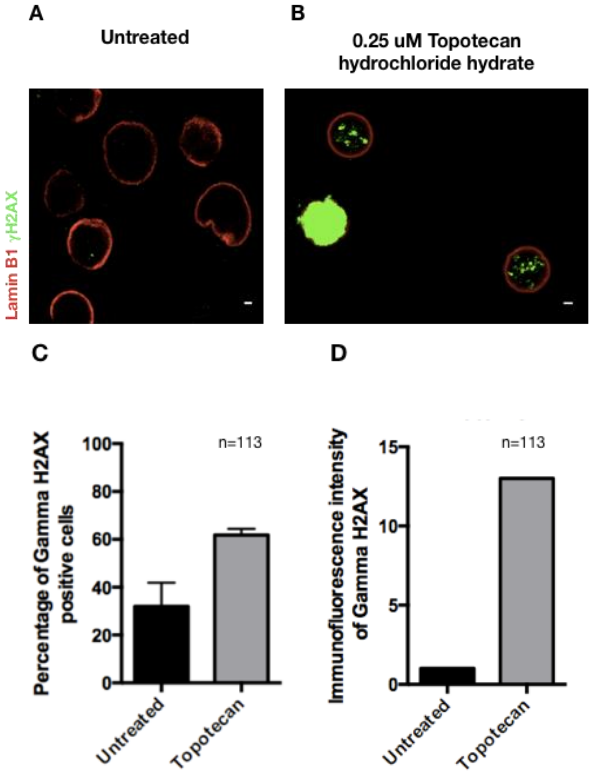


Figure 4. Immunofluorescence staining of γH2AX DNA damage marker in untreated and topotecan treated primary CD4$^+$ T cells. A: γH2AX staining in untreated primary CD4$^+$ T cells; B: γH2AX staining in 0,25μM topotecan treated primary CD4$^+$ T cells, 7 hours post drug treatment; C:Quantification of γH2AX positive cells in untreated and topotecan treated conditions. In total, 113 cells were analyzed. D: Total immunofluorescence intensity of γH2AX in untreated and topotecan treated conditions. In total, 113 cells were analyzed.

Previous studies have linked the cytotoxic effects of topotecan with increased DNA damage and consequent apoptosis (Bertrand et al., 1994). To test whether this mechanism of action was responsible for the massive cell death detected in CD4$^+$ T cells,

we stained for the DNA double strand break marker γH2AX (Figure 4).

The results show a marked increase in γH2AX signals which almost double in number (from 31,98% to 61,82% of positive cells) upon treatment with topotecan (Figure 4). The observed difference is even more significant when comparing the total immunofluorescence intensity of γH2AX signals in untreated and topotecan treated cells. Topotecan treated cells show a 13 fold increase in total immunofluorescence signal of γH2AX compared to untreated cells, indicating the presence of severe DNA damage and ongoing apoptosis.

Consistently with these results, 72 hours post drug removal all analyzed cell nuclei were either fully covered with γH2AX signals, or reduced to cell debris, as a result of advanced cell death (data not shown).

Conclusions

Overall, these data prove that DNA damage and cell death in primary $CD4^+$ T cells start already 3 hours post topotecan treatment and quickly progresses to complete cell death at all tested concentrations. Our results suggest that for experimental purposes the administration of small concentrations of topotecan for very short time intervals could be sufficient to achieve the desired effect of topoisomerase-I inhibition. Moreover, obtained results point to potentially severe toxicity on activated T-lymphocytes, further confirming the need to regulate the amounts of administered drug to control its efficacy and minimize cytotoxicity, as previously described (Hassan et al., 2001; Cordell, 2003). Finally, as already reported, our data show that interfering with the activity of topoisomerases provokes genomic instability and could lead to secondary malignancies (Pommier et al., 2016) (especially during cancer therapy) as a consequence of nonselective targeting of healthy cells.

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