

Original Paper

Triggering of Suicidal Erythrocyte Death by Topotecan

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Key Words

Phosphatidylserine • Cell volume • Ceramide • Calcium • Oxidative stress • Eryptosis

Abstract

Background/Aims: The topoisomerase I inhibitor topotecan is used as treatment of various malignancies. The substance is effective by triggering tumor cell apoptosis. In analogy to apoptosis of nucleated cells, erythrocytes may enter eryptosis, a suicidal death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the outer face of the erythrocyte membrane. Signaling leading to eryptosis include Ca^{2+} -entry and ceramide formation. The present study explored, whether and how topotecan induces eryptosis. **Methods:** Phosphatidylserine abundance at the erythrocyte surface was estimated from annexin V binding, cell volume from forward scatter, and ceramide abundance utilizing specific antibodies. **Results:** A 48 hours exposure of human erythrocytes to topotecan significantly increased the percentage of annexin-V-binding cells and significantly decreased forward scatter. The effect of topotecan was paralleled by a significant increase of ceramide abundance. The effect of topotecan on annexin-V-binding was significantly blunted, but not abolished by removal of extracellular Ca^{2+} . **Conclusions:** Topotecan stimulated cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect paralleled by increase of ceramide abundance and partially dependent on entry of extracellular Ca^{2+} .

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Introduction

The camptothecin analogue [1] topotecan is utilized for the treatment of malignancy, such as ovarian cancer [2-7], cervical cancer [5, 8-10], retinoblastoma [11-13], small cell lung cancer [14-18], hematopoietic cell malignancy [19, 20] and Wilms' tumor [21]. It is applied at a dosage of 10 mg/kg b.w. in mice and reaches plasma concentrations of up to 180 $\mu\text{g}/\text{mL}$ [22]. In humans, the maximally tolerated topotecan dose is 0.75 mg/m² which results

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in a mean peak plasma concentration of 2.74 ng/mL [23]. Side effects of topotecan include thrombocytopenia, neutropenia, alopecia, fatigue, diarrhea, nausea and anemia [24].

Topotecan is effective against tumor cells in part by triggering apoptosis [1, 25-33]. The cytotoxicity of topotecan has been attributed to inhibition of topoisomerase I [1, 33-37], double-stranded DNA damage [8, 33], down-regulation of the transcription factors MycN [38], NF- κ B [30] and HIF-1 α [36], suppression of PTEN [32], VEGF [36] and Bcl-2 expression [31, 37], oxidative stress [25], decrease of Akt activity [36], as well as caspase activation [25-27, 33, 37].

In analogy to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage [39] and break down of phospholipid asymmetry of the cell membrane leading to phosphatidylserine translocation to the cell surface [40]. Signaling triggering eryptosis include Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [40], ceramide [41], energy depletion [40], oxidative stress [40], caspase activation [40, 42, 43], stimulation of casein kinase 1 α [44, 45], Janus-activated kinase JAK3 [46], protein kinase C [47] and p38 kinase [48]. Eryptosis is inhibited by PAK2 kinase [49], AMP activated kinase AMPK [50], cGMP-dependent protein kinase [51] and sorafenib/sunitinib sensitive kinases [52, 53]. Eryptosis is triggered by a myriad of xenobiotics [40, 54-84].

The present study explored, whether and how topotecan triggers eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to Ringer without or with topotecan and cell volume as well as phosphatidylserine and ceramide abundance determined by flow cytometry.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 x g for 20 min at 21°C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, pH 7.4), 5 glucose, and 1 CaCl_2 ; at 37°C for 48 hours. Where indicated, erythrocytes were exposed to topotecan (Tocris Cookson Ltd., Bristol, UK) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 μ l cell suspension was washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. The annexin V abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin V binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin V-binding cells and control cells. The same threshold was used for untreated and topotecan treated erythrocytes. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of "52", i.e. values below 52 were not considered.

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl_2 and 5 μ M Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μ l Ringer. Then, Ca^{2+} -dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. No lower threshold of Fluo3 fluorescence was set.

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, a 100 μ l cell suspension was stained for 1 hour at 37°C with 1 μ g/mL anti-ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. Subsequently, the cells were stained for 30 minutes with polyclonal fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were then analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 μ l suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnellendorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 μ M. Erythrocytes were incubated at 37°C for 30 min in the dark and then washed three times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μ l Ringer solution, and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Statistics

Data are expressed as arithmetic means \pm SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and t test as appropriate; n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study tested, whether topotecan modifies suicidal erythrocyte death or eryptosis. To this end, the hallmarks of eryptosis, i.e. cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface, were determined utilizing flow cytometry.

Cell volume was estimated from forward scatter. As illustrated in Fig. 1, exposure of erythrocytes for 48 hours to Ringer solution with 125-175 μ g/mL topotecan was followed by a marked significant decrease of forward scatter reflecting cell shrinkage.

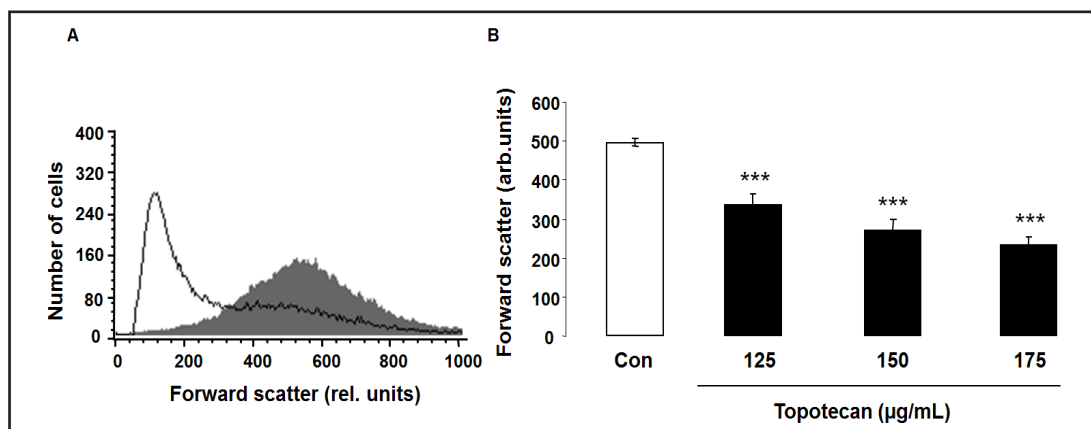


Fig. 1. Effect of topotecan on erythrocyte forward scatter. (A) Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 175 μ g/mL topotecan. (B) Arithmetic means \pm SEM (n = 8) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) topotecan (125-175 μ g/mL). *** (P < 0.001) indicates significant difference from the absence of topotecan (ANOVA).

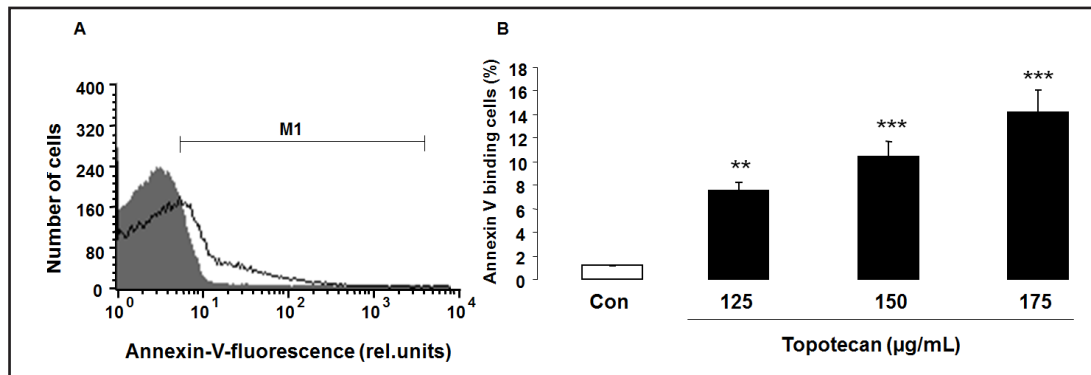


Fig. 2. Effect of topotecan on phosphatidylserine exposure. (A) Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 175 µg/mL topotecan. (B) Arithmetic means ± SEM (n = 8) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) topotecan (125-175 µg/mL). ** (P < 0.01), *** (P < 0.001) indicate significant difference from the absence of topotecan (ANOVA).

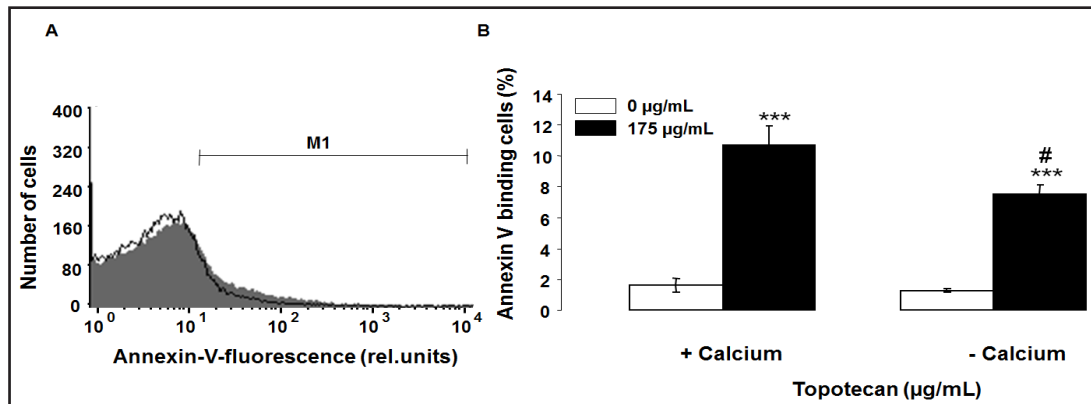


Fig. 3. Ca²⁺ sensitivity of topotecan induced translocation of phosphatidylserine exposure. (A) Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution in the absence of extracellular Ca²⁺ without (grey area) and with (black line) presence of 175 µg/mL topotecan. (B) Arithmetic means ± SEM (n = 10) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with 175 µg/mL topotecan (black bars) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. *** (P < 0.001) indicates significant difference from the absence of topotecan, # (P < 0.05) indicates significant difference from the presence of Ca²⁺ (ANOVA).

Phosphatidylserine at the erythrocyte surface was quantified utilizing annexin-V-binding. As shown in Fig. 2, exposure of erythrocytes for 48 hours to Ringer solution with 125-175 µg/mL topotecan was followed by a marked significant increase of the percentage of annexin-V-binding erythrocytes reflecting an increase of phosphatidylserine exposing erythrocytes.

Further experiments were performed in order to shed light on the signaling mediating topotecan induced eryptosis. Attempts were made to determine cytosolic Ca²⁺ activity ([Ca²⁺]_i) with Fluo3 fluorescence. However, topotecan treatment was followed by a decrease of Fluo3 fluorescence in the presence of Ca²⁺ ionophore ionomycin (1 µM 60 min) pointing to loss of fluorescent dye. Possibly, topotecan inhibits the cytosolic esterases or permeabilizes the cell membrane for the dye. Alternatively the average was decreased by Fluo3 containing vesicles. In any case, the effect precluded safe conclusions regarding the effect of topotecan on [Ca²⁺]_i.

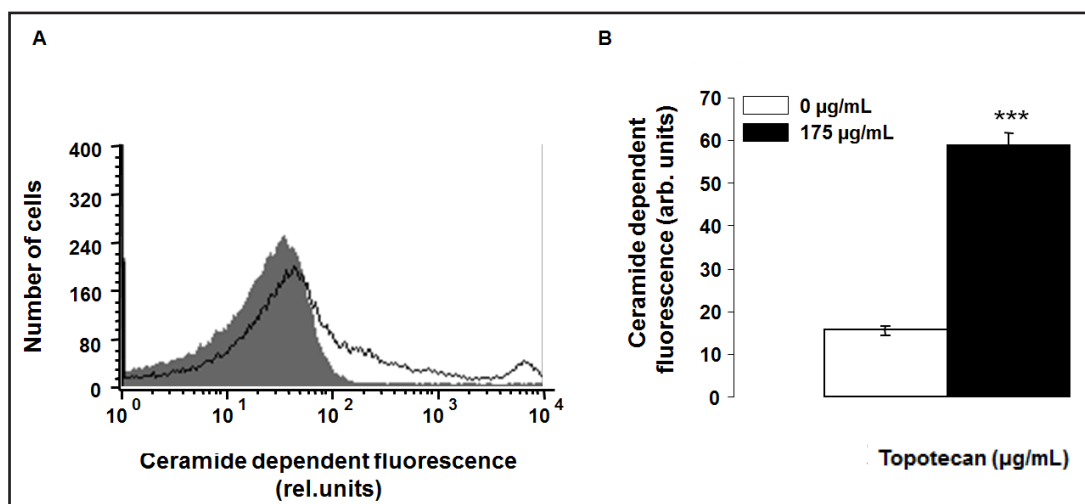


Fig. 4. Effect of topotecan on ceramide abundance. (A) Original histogram of ceramide abundance at the erythrocyte surface following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 175 µg/mL topotecan. (B) Arithmetic means \pm SEM ($n = 5$) of ceramide abundance at the erythrocyte surface following incubation for 48 hours to Ringer solution without (white bar) or with 175 µg/mL topotecan (black bar). *** ($P < 0.001$) indicates significant difference from the absence of topotecan (t test).

In order to test whether the effect of topotecan on cell volume and/or phosphatidylserine translocation required entry of extracellular Ca^{2+} , forward scatter and annexin-V-binding were determined following exposure to topotecan (175 µg/mL) in the presence and in the absence of extracellular Ca^{2+} . As a result, topotecan exposure was followed by a significant decrease of forward scatter to similar values in the presence and in the absence of extracellular Ca^{2+} . In the presence of Ca^{2+} , topotecan decreased the forward scatter from 505 ± 7 to 266 ± 26 ($n = 10$) and in the absence of Ca^{2+} , topotecan decreased the forward scatter from 506 ± 8 to 254 ± 24 ($n = 10$). No significant differences were observed between presence and absence of Ca^{2+} . Accordingly, entry of Ca^{2+} did not account for the stimulation of cell shrinkage following topotecan exposure. As illustrated in Fig. 3, topotecan exposure was followed by a significant increase of the percentage of annexin-V-binding erythrocytes both, in the presence and in the absence of extracellular Ca^{2+} . The effect was, however, significantly blunted in the absence of extracellular Ca^{2+} . Accordingly, topotecan-induced cell membrane scrambling was partially but not fully dependent on entry of Ca^{2+} .

Stimulators of eryptosis without requirement of increased $[\text{Ca}^{2+}]_i$ include ceramide. In order to test, whether topotecan influences ceramide abundance at the erythrocyte surface, ceramide was visualized with specific antibodies. As a result, a 48 hours incubation with topotecan (175 µg/mL) significantly increased the ceramide abundance (Fig. 4).

Triggers of eryptosis further include oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As a result, following a 48 hours incubation the DCFDA fluorescence was rather lower in the presence (12.2 ± 0.9 a.u., $n = 5$) than in the absence (16.9 ± 2.4 a.u., $n = 5$) of topotecan (175 µg/mL), a difference, however, not reaching statistical significance. For comparison, a 10 mins exposure of erythrocytes to 0.3 mM *tert*-butylhydroperoxide (TBOOH) was followed by a significant increase of DCFDA fluorescence (182.6 ± 10.8 a.u., $n = 4$).

Eryptosis is further stimulated by caspases. In order to test whether the effect of topotecan on phosphatidylserine translocation required caspase activity, annexin-V-binding was determined following exposure to topotecan (175 µg/mL) in the presence and in the absence of caspase inhibitor zVAD (10 µM). As a result, topotecan exposure was followed by a significant increase of annexin-V-binding to similar values in the absence and presence of zVAD. Topotecan increased the percentage of annexin-V-binding erythrocytes from $3.0 \pm$

0.4 to 17.0 ± 0.6 ($n = 5$) in the absence of zVAD and from 3.6 ± 0.4 to 16.5 ± 0.6 ($n = 5$) in the presence of zVAD. No significant differences were observed between presence and absence of zVAD.

Discussion

The present observations uncover a novel action of topotecan, i.e. the stimulation of eryptosis, the suicidal erythrocyte death. Eryptosis is characterized by the two hallmarks cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Topotecan exposure was followed by both, decrease of forward scatter reflecting cell shrinkage and by increase of annexin-V-binding, reflecting phosphatidylserine abundance at the cell surface. The concentrations required are in the range of those reported in plasma of mice treated with liposome-encapsulated topotecan [22]. Concentrations effective in the treatment of tumors, are however, lower, i.e. in the range of 2.7 ng/mL to 0.21 μ g/mL [23, 85]. It must be kept in mind, though, that the susceptibility to triggers of eryptosis is enhanced in several clinical conditions, such as dehydration [70], hyperphosphatemia [81], chronic kidney disease (CKD) [59, 86-88], hemolytic-uremic syndrome [89], diabetes [90], liver failure [91], malignancy [92], sepsis [93] and Wilsons disease [94].

Topotecan triggered cell membrane scrambling was in part dependent on presence of extracellular Ca^{2+} and presumably required in part Ca^{2+} entry. An increase of $[Ca^{2+}]_i$ stimulates cell membrane scrambling [40] and activates Ca^{2+} sensitive K^+ channels with subsequent cell shrinkage due to K^+ exit, cell membrane hyperpolarization, and Cl^- exit, which lead to cellular loss of KCl with osmotically obliged water [39]. Removal of extracellular Ca^{2+} did, however, not significantly modify topotecan-induced cell shrinkage and only partially blunted cell membrane scrambling pointing to involvement of additional cellular mechanisms.

The stimulation of cell membrane scrambling was paralleled by increase of ceramide abundance at the cell surface. Ceramide is a well known trigger of eryptosis even in the absence of increased $[Ca^{2+}]_i$ [40].

The stimulation of cell membrane scrambling was not paralleled by increase of reactive oxidant species (ROS), which is another well known trigger of eryptosis [40].

Eryptosis accomplishes the clearance of defective erythrocytes prior to hemolysis [40], thus preventing release of hemoglobin, which is filtered in renal glomeruli, precipitates in the acidic lumen of renal tubules and thus occludes nephrons [95]. Eryptosis further leads to clearance of infected erythrocytes in malaria, and may thus favourably influence the clinical course of the disease [96]. The malaria pathogen *Plasmodium* imposes oxidative stress on the infected host erythrocyte with subsequent activation of host cell ion channels including Ca^{2+} -permeable erythrocyte cation channels [40, 97]. The Ca^{2+} -entry triggers eryptosis with subsequent clearance of the infected erythrocytes [96]. Enhanced susceptibility to eryptosis thus protects against a severe course of the disease. Accordingly, sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency protect against a severe course of malaria [40, 98-100]. Eryptosis is further triggered and thus the course of malaria favourably influenced by iron deficiency [101] and treatment with lead [101], chlorpromazine [102] or NO synthase inhibitors [102].

The clearance of phosphatidylserine exposing erythrocytes from circulating blood following eryptosis may lead to anemia [40]. Moreover, phosphatidylserine exposing erythrocytes adhere to the vascular wall [103], trigger blood clotting and elicit thrombosis [104-106]. Stimulation of eryptosis may thus lead to impairment of microcirculation [41, 104, 107-110].

Conclusion

Topotecan triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect involving Ca^{2+} entry and increase of ceramide abundance.

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Disclosure Statement

The authors declare that they have nothing to disclose.

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