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Original Paper

Mitoxantrone-Induced Suicidal Erythrocyte Death

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Key Words
Phosphatidylserine • Mitoxantrone • Calcium • Ceramide • Cell volume • Eryptosis

Abstract
Background/Aims: Mitoxantrone, a cytotoxic drug used for the treatment of malignancy and multiple sclerosis, is at least in part effective by triggering apoptosis. Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, a type of suicidal cell death. Hallmarks of eryptosis are cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signalling involved in eryptosis include Ca^{2+}-entry, ceramide formation and oxidative stress. ***Methods:*** Cell volume was estimated from forward scatter, phosphatidylserine-exposure from annexin V binding, formation of reactive oxidant species (ROS) from 2′,7′-dichlorodihydrofluorescein-diacetate fluorescence, and ceramide abundance from binding of fluorescent antibodies in flow cytometry. ***Results:*** A 48 hours exposure to mitoxantrone was followed by significant decrease of forward scatter (≥ 5 µg/ml mitoxantrone) and increase of annexin-V-binding (≥ 10 µg/ml mitoxantrone), effects paralleled by significant increases of ROS formation (25 µg/ml mitoxantrone) and ceramide abundance (25 µg/ml mitoxantrone). The effect of mitoxantrone was not significantly modified by nominal absence of extracellular Ca^{2+} but significantly blunted by the antioxidant N-acetylcysteine (1 mM). ***Conclusions:*** Mitoxantrone triggers cell membrane scrambling, an effect not requiring entry of extracellular Ca^{2+} but at least partially due to formation of ROS and ceramide.

Introduction

Mitoxantrone, a synthetic antineoplastic cytotoxic drug [1], is used for the treatment of both, aggressive multiple sclerosis [1, 2] and malignancy [3-10]. The antineoplastic effects result from triggering of tumor cell apoptosis [11-17], the efficacy of mitoxantrone in multiple sclerosis has been attributed to apoptosis of lymphocytes and dendritic cells [18].

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Furthermore, mitoxantrone may induce cell senescence [19]. Side effects of mitoxantrone include leukemia [20], cardiotoxicity [21], anemia [21] and thrombosis [22-27].

In analogy to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal erythrocyte death. Hallmarks of eryptosis are cell shrinkage and cell membrane scrambling with phosphatidylserine exposure to the erythrocyte surface [28]. Triggers of eryptosis include entry of Ca^{2+} with subsequent increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [29], oxidative stress [28], ceramide formation [28], caspase activation [30-34], knockout of AMP activated kinase AMPK [35], or of cGMP-dependent protein kinase [36], inhibition of p21 activated kinase [37] or sorafenib [38] and sunitinib [39] sensitive kinases and/or activation of casein kinase 1α [40, 41], Janus-activated kinase JAK3 [42], protein kinase C [43] or p38 kinase [44].

The present study tested, whether mitoxantrone stimulates eryptosis, and explored the impact of putative underlying mechanisms, such as Ca^{2+} entry, oxidative stress, p38 kinase and ceramide formation.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Lithium-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 (184/2003V). The blood was centrifuged at 120 rcf for 20 minutes at 23°C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were washed in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4. For the experiments, erythrocytes were incubated *in vitro* at a hematocrit of 0.4% at 37°C for 48 hours. Where indicated, erythrocytes were exposed to mitoxantrone (Sigma-Aldrich, Hamburg, Germany) at the indicated concentrations. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). Where indicated, the antioxidant N-acetylcysteine (1 mM) or the p38 kinase blocker SB 203580 (2 μM) was added.

FACS analysis of annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 50 μl cell suspension was washed in Ringer solution containing 5 mM $CaCl_2$ to provide Ca^{2+} for Ca^{2+}-dependent Annexin-V-FITC-binding. Cells were then stained with Annexin-V- fluorescein isothiocyanate (FITC) (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. In the following, the forward scatter (FSC) of the cells was determined, and annexin-V-FITC fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS-calibur from Becton Dickinson (Heidelberg, Germany).

Measurement of 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 μM 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 μl 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.

Determination of oxidative stress

Reactive oxygen species (ROS) production was determined utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) [45]. Briefly, the cells were suspended in Ringer solution and the fluorescence was analysed with flow cytometry (FACS-calibur). DCFDA fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

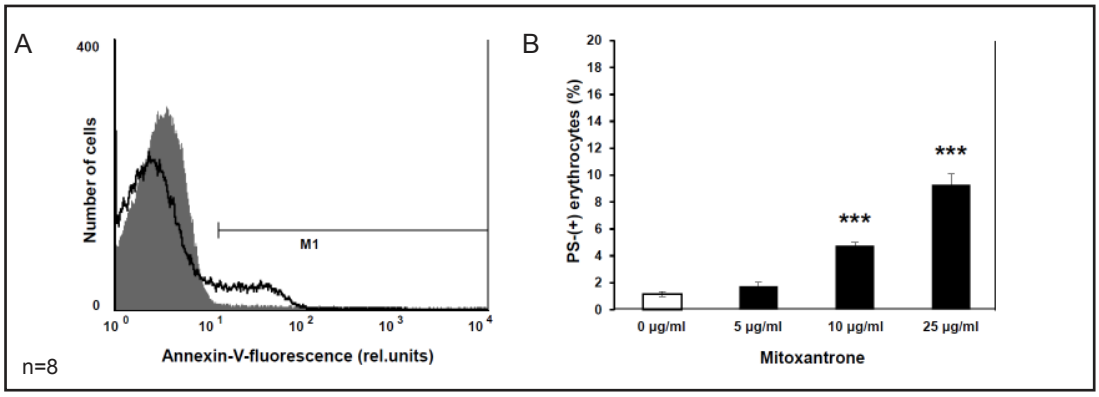


Fig. 1. Effect of mitoxantrone on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes (M1) following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 25 µg/ml mitoxantrone. B. Arithmetic means ± SEM (n = 8) of annexin-V-binding (i.e. phosphatidylserine-positive, PS-(+)) erythrocytes in % following incubation for 48 hours to Ringer solution without (1µg/ml DMSO alone, white bar) or with (black bars) presence of mitoxantrone (5-25 µg/ml in 1µg/ml DMSO). *** ($p<0.001$) indicates significant difference from the absence of mitoxantrone [presence of DMSO alone] (ANOVA).

Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 hour at 37°C with 0.1 µg/ml anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) diluted in PBS containing 0.1% bovine serum albumin (BSA). The samples were washed twice with PBS-BSA. Subsequently, the cells were stained for 30 minutes with polyclonal 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.

Measurement of hemolysis

For the determination of hemolysis, the samples were centrifuged (3 min at 1600 rpm, room temperature) after incubation under the above mentioned experimental condition, the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using one-way ANOVA with Tukey's test as post-test and two-tailed *t*-test as appropriate. The number of different erythrocyte specimens studied is given as n. 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 explored, whether mitoxantrone triggers eryptosis, the suicidal erythrocyte death. The hallmarks of eryptosis include cell membrane scrambling with phosphatidylserine translocation to the cell surface. Annexin-V-binding was quantified by flow cytometry in order to identify phosphatidylserine exposing erythrocytes. As illustrated in Fig. 1, a 48 hours treatment with mitoxantrone was followed by an increase of the percentage annexin-V-binding erythrocytes, an effect reaching statistical significance at 10 µg/ml mitoxantrone concentration.

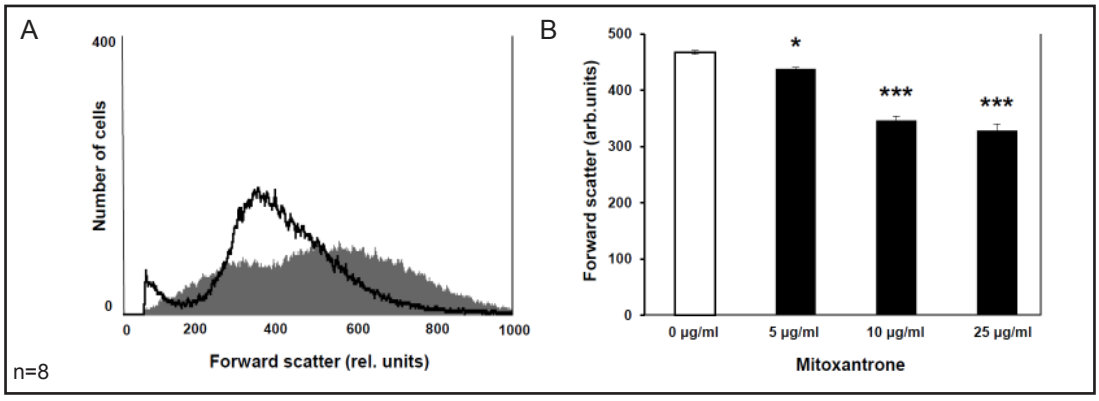
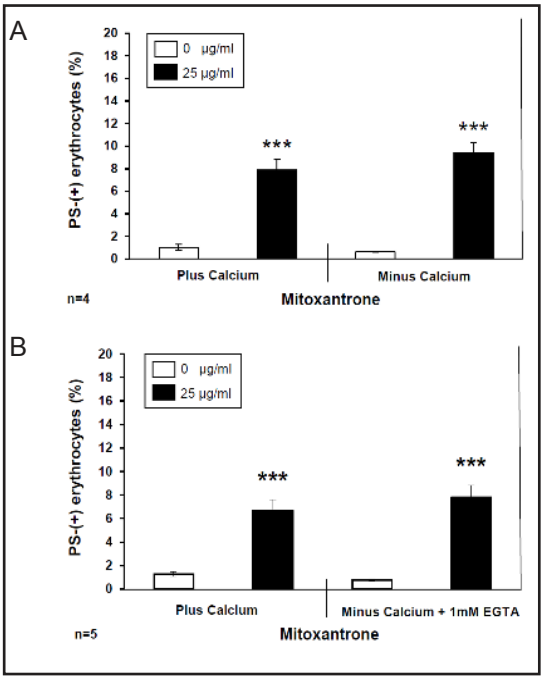


Fig. 2. Effect of mitoxantrone on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 25 µg/ml mitoxantrone. B. Arithmetic means ± SEM (n = 8) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (1µg/ml DMSO alone, white bar) or with (black bars) mitoxantrone (5-25 µg/ml in 1µg/ml DMSO). * ($p<0.05$), *** ($p<0.001$) indicate significant differences from the absence of mitoxantrone [presence of DMSO alone] (ANOVA).

Fig. 3. Effect of mitoxantrone in the presence and absence of extracellular Ca^{2+}. A,B Arithmetic means ± SEM (n = 4-5) of the percentage of annexin-V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) 25 µg/ml mitoxantrone in the presence (left bars, Plus Calcium) and absence (right bars, Minus Calcium) of calcium without (A) and with (B) EGTA added. *** ($p<0.001$) indicates significant difference from the respective values in the absence of mitoxantrone (ANOVA).



Erythrocyte death could involve hemolysis, a cell death distinct from eryptosis. In order to determine, whether mitoxantrone triggers hemolysis, the percentage of hemolysed erythrocytes was quantified from hemoglobin concentration in the supernatant. As a result, following a 48 hours incubation with 0, 5, 10 and 25 µg/ml mitoxantrone, respectively, the hemoglobin concentration in the supernatant approached 1.3 ± 0.6 %, 1.5 ± 0.2 %, 5.9 ± 2.5 %, and 8.5 ± 0.8 % [**] of hemoglobin concentration following complete hemolysis (n = 5). The difference between presence and absence of mitoxantrone reached statistical significance ($p<0.01$) at 25 µg/ml mitoxantrone concentration (ANOVA).

Eryptosis is further characterized by cell shrinkage. Accordingly, cell volume was estimated from forward scatter in flow cytometry. As illustrated in Fig. 2, a 48 hours treatment with mitoxantrone decreased forward scatter, an effect reaching statistical significance at 5 µg/ml mitoxantrone concentration.

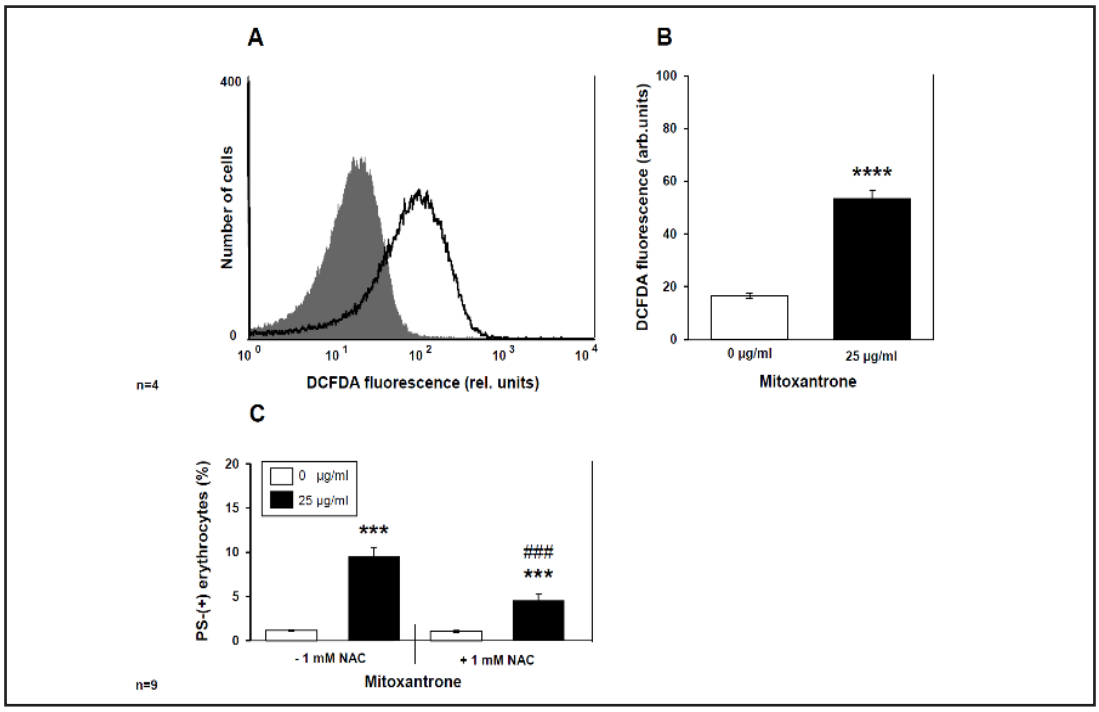


Fig. 4. Effect of mitoxantrone on reactive oxygen species. A. Original histogram of DCFDA-fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 25 µg/ml mitoxantrone. B. Arithmetic means ± SEM (n = 4) of erythrocyte DCFDA fluorescence following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) presence of mitoxantrone (25 µg/ml). **** ($p<0.0001$) indicates significant difference from the absence of mitoxantrone (two-tailed t-test). C. Arithmetic means ± SEM (n = 9) of the percentage of annexin-V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) 25 µg/ml mitoxantrone in the absence (left bars, -NAC) and presence (right bars, +NAC) of the antioxidant N-acetylcysteine (1 mM). *** ($p<0.001$); indicates significant difference from the respective values in the absence of mitoxantrone (ANOVA), ### ($p<0.001$) indicates significant difference from the respective values in the absence of N-acetylcysteine (ANOVA).

Cell membrane scrambling and cell shrinkage following mitoxantrone treatment could have been due to increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) resulting from entry of extracellular Ca^{2+}. Attempts to quantify $[Ca^{2+}]_i$ in erythrocytes utilizing Fluo3 fluorescence failed, as the cells apparently lost the dye following mitoxantrone treatment (data not shown). Possibly, mitoxantrone compromizes cell membrane integrity leading to hemolysis on the one hand and loss of Fluo3 on the other. In order to test, whether the effect of mitoxantrone on cell membrane scrambling required the entry of extracellular Ca^{2+}, erythrocytes were exposed for 48 hours to 25 µg/ml mitoxantrone in the presence or nominal absence of extracellular Ca^{2+}. As shown in Fig. 3, the effect of mitoxantrone on annexin-V-binding was not significantly different between the nominal absence and the presence of Ca^{2+}. Mitoxantrone significantly increased the percentage of annexin-V-binding erythrocytes in both, the absence and presence of extracellular Ca^{2+}. This result indicates that the effect of mitoxantrone on cell membrane scrambling did not require Ca^{2+} entry.

Further experiments addressed alternative mechanisms possibly involved in the effect of mitoxantrone on eryptosis. The ROS-formation was determined utilizing DCFDA. As illustrated in Fig. 4A,B, a 48 hours exposure to 25 µg/ml mitoxantrone markedly and significantly increased the DCFDA fluorescence, a finding pointing to induction of oxidative stress. In order to test, whether ROS was required for the full effect of mitoxantrone on cell membrane scrambling, erythrocytes were exposed for 48 hours to 25 µg/ml mitoxantrone in the absence and presence of the antioxidant N-acetylcysteine (1 mM). As illustrated in Fig.

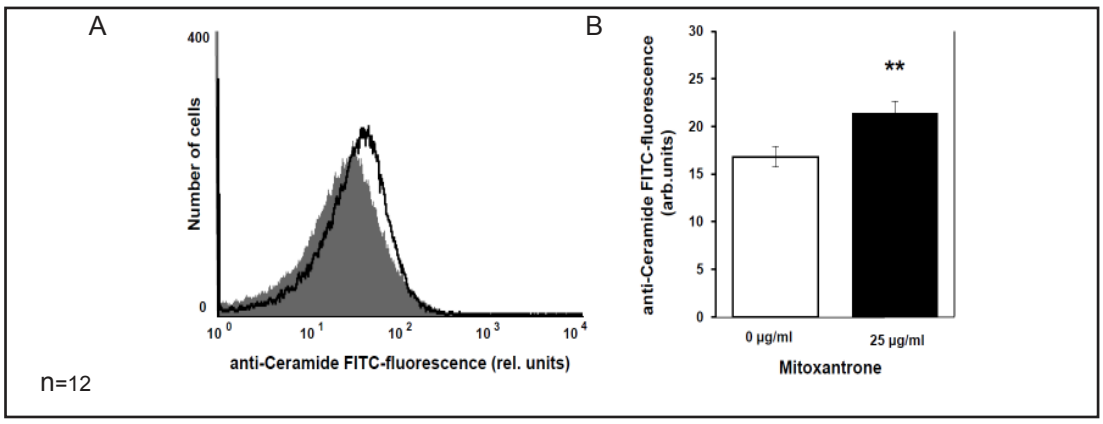


Fig. 5. Effect of mitoxantrone on ceramide abundance. A. Original histogram of anti-ceramide FITC fluorescence in erythrocytes after exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 25 µg/ml mitoxantrone. B. Arithmetic means ± SEM (n = 12) of ceramide abundance at the erythrocyte surface following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) presence of mitoxantrone (25 µg/ml). ** ($p<0.01$) indicates significant difference from the absence of mitoxantrone (two-tailed t-test)

4C, the effect of mitoxantrone on annexin-V-binding was significantly blunted in the presence of N-acetylcysteine. This result indicates that the effect of mitoxantrone on cell membrane scrambling was at least partially due to oxidative stress.

In order to test, whether the effect of mitoxantrone required activation of p38 kinase, experiments were performed in the absence and presence of the p38 kinase inhibitor SB203580 (2 µM). As a result, a 48 hours exposure to 25 µg/ml mitoxantrone increased the percentage of annexin-V-binding erythrocytes significantly from 1.7 ± 0.3 % to 11.3 ± 0.8 % in the absence of SB203580 and significantly from 1.9 ± 0.4 % to 11.2 ± 0.8 % in the presence of SB203580. The increase was similar in the absence and presence of SB203580 indicating that activation of p38 kinase was not required for the stimulation of eryptosis by mitoxantrone.

As cell membrane scrambling could further be triggered by ceramide, additional experiments tested, whether mitoxantrone increases the ceramide abundance at the erythrocyte surface. The abundance of ceramide at the erythrocyte surface was quantified utilizing anti-ceramide antibodies. As illustrated in Fig. 5, a 48 hours exposure to 25 µg/ml mitoxantrone was followed by a significant increase of ceramide abundance at the erythrocyte surface.

Discussion

The present study discloses a novel effect of mitoxantrone, i.e. stimulation of eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and breakdown of phosphatidylserine asymmetry of the erythrocyte cell membrane. The mitoxantrone concentrations required for the stimulation of eryptosis are within the range of mitoxantrone concentrations required for the antineoplastic activity of the substance [46]. At least in theory, the erythrocytes could be sensitized to the effect of mitoxantrone by parallel exposure to further eryptosis triggering xenobiotics [28, 39, 47-73]. Moreover, the sensitivity to mitoxantrone may be increased in patients suffering from disorders with enhanced eryptosis [28], such as diabetes [34, 74, 75], dehydration [76], renal insufficiency [77, 78], hemolytic uremic syndrome [79], sepsis [80], malaria [81], sickle cell disease [81], Wilson's disease [82], iron deficiency [83], malignancy [84], phosphate depletion [85], and metabolic syndrome [65].

The effect of mitoxantrone did not require Ca^{2+} entry. Whether or not mitoxantrone modifies cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), cannot be answered with certainty. The exposure to

mitoxantrone was followed by a decline of Fluo3 fluorecence, which may result from decrease of $[Ca^{2+}]_i$ or from loss of dye. Whatever effect mitoxantrone exerts on $[Ca^{2+}]_i$, the effect appears not to be relevant for the triggering of eryptosis. We thus explored further putative mechanisms. As evident from DCFDA fluorescence, mitoxantrone induces oxidative stress, a well known trigger of eryptosis [28]. Mitoxantrone has previously been shown to similarly induce oxidative stress of nucleated cells [8, 86-88]. On the other hand, mitoxantrone may strengthen the antioxidative defence [89, 90]. The latter effect presumably requires expression of the respective proteins, an effect not possible in erythrocytes. In erythrocytes oxidative stress activates Ca^{2+} permeable cation channels with subsequent Ca^{2+} entry [91], an effect apparently not critically important for the stimulation of cell membrane scrambling by mitoxantrone. Oxidative stress further activates erythrocyte Cl^- channels and aspartyl and cysteinyl proteases [91]. Additional experimentation will be required to fully elucidate the link between mitoxantrone induced oxidative stress and cell membrane scrambling.

Mitoxantrone further increased the abundance of ceramide, another powerful stimulator of eryptosis [28]. Mitoxantrone may stimulate ceramide formation by activation of sphingomyelinase in nucleated cells [92]. Mitoxantrone has been shown to stimulate phospholipase C with subsequent hydrolysis of phosphatidylcholine [92]. In erythrocytes, sphingomyelinase and thus ceramide formation are stimulated by platelet activating factor [93, 94]. Ceramide has been shown to trigger cell membrane scrambling in part by sensibilisation against Ca^{2+} [93]. The present observations rather suggest Ca^{2+} independence of mitoxantrone-induced eryptosis.

Eryptosis is a physiological mechanism accomplishing removal of defective erythrocytes from circulating blood. Phosphatidylserine exposing erythrocytes bind to respective receptors of phagocyting cells which engulf and degrade the defective erythrocytes [28]. Accordingly, phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood [28]. Stimulation of eryptotic cell shrinkage [29] counteracts erythrocyte swelling, which could, at least in theory, result in hemolysis with subsequent rupture of the cell membrane and release of cellular hemoglobin. Hemoglobin released from hemolytic erythrocytes may be filtered in renal glomeruli, undergo subsequent precipitation in renal tubules, occlude tubular lumina and thus lead to obstructive kidney injury [95].

Excessive eryptosis may be similarly harmful. Removal of phosphatidylserine exposing erythrocytes from circulating blood may result in anemia, as soon as the loss of eryptotic erythrocytes exceeds the parallel formation of new erythrocytes [28]. Phosphatidylserine exposing erythrocytes furher adhere to the vascular wall by binding of erythrocytic phosphatidylserine to endothelial CXCL16 [96]. The binding of eryptotic erythrocytes to the vascular wall impedes microcirculation [96-101]. Phosphatidylserine exposing erythrocytes may further foster blood clotting and thus thrombosis [97, 102, 103].

In conclusion, mitoxantrone triggers eryptosis with erythrocyte shrinkage and cell membrane scrambling. Cellular mechanisms involved in the pro-eryptotic effect of mitoxantrone include oxidative stress and ceramide formation.

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