

Original Paper

Edelfosine Induced Suicidal Death of
Human ErythrocytesMarilena Briglia^{a,b} Antonella Fazio^b Elena Signoretto^c Caterina Faggio^b
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Key Words

Phosphatidylserine • Cell volume • Eryptosis • Red blood cell • Calcium

Abstract

Background/Aims: The anti-inflammatory, anti-autoimmune, antiparasitic, and anti-viral ether phospholipid edelfosine (1-O-octadecyl-2-O-methylglycero-3-phosphocholine) stimulates apoptosis of tumor cells and is thus considered for the treatment of malignancy. Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the erythrocyte surface. Triggers of eryptosis include Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) and oxidative stress. The present study explored, whether and how edelfosine induces eryptosis. **Methods:** Flow cytometry and photometry, respectively, were employed to estimate phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, hemolysis from hemoglobin release, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, and abundance of reactive oxygen species (ROS) from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. **Results:** A 6 hours exposure of human erythrocytes to edelfosine (5 μM) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter, and significantly increased Fluo3-fluorescence, but did not significantly modify DCFDA fluorescence. The effect of edelfosine on annexin-V-binding was significantly blunted, but not abolished by removal of extracellular Ca^{2+} . **Conclusions:** Edelfosine triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part due to stimulation of Ca^{2+} entry.

Introduction

The anti-inflammatory [1], anti-autoimmune [2, 3], antiparasitic [4-8] and anti-viral [9] ether phospholipid edelfosine (1-O-octadecyl-2-O-methylglycero-3-phosphocholine) triggers apoptosis of tumor cells and is thus considered for the treatment of malignancy

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[10-35]. Mechanisms involved in edelfosine-induced apoptosis include formation of plasma membrane lipid rafts recruiting death receptor and downstream apoptotic signaling molecules [14, 16, 19, 20, 24, 25, 27], endoplasmic reticulum (ER) stress response [13, 17, 20, 29, 30], mitochondrial depolarisation [20, 25, 35], cytochrome c release [13], caspase activation [3, 13, 16, 23] and generation of reactive oxygen species [13, 31, 35].

Even though lacking mitochondria and nuclei, key organelles in the execution of apoptosis, erythrocytes may - similar to nucleated cells - enter suicidal death or eryptosis [36], which is characterized by cell shrinkage [37] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [36]. Triggers of eryptosis include opening of oxidant sensitive Ca^{2+} permeable unselective cation channels with subsequent Ca^{2+} entry and increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [36]. Eryptosis is further stimulated by ceramide [38], energy depletion [36], caspases [36, 39, 40], casein kinase 1 α [36], Janus-activated kinase JAK3 [36], protein kinase C [36], and p38 kinase [36]. Eryptosis is inhibited by AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase, and sorafenib/sunitinib sensitive kinases [36]. Eryptosis is stimulated by a large number of diverse xenobiotics [36, 41-65] and is accelerated in several clinical conditions, such as dehydration [54], hyperphosphatemia [64] chronic kidney disease (CKD) [46, 66-68], hemolytic-uremic syndrome [69], diabetes [70], hepatic failure [71], malignancy [36], sepsis [72], sickle-cell disease [36], beta-thalassemia [36], Hb-C-deficiency [36], G6PD-deficiency [36], and Wilsons disease [73].

The present study explored whether and how edelfosine triggers eryptosis. To this end, human erythrocytes from healthy volunteers were treated with edelfosine and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, as well as abundance of reactive oxygen species (ROS) 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 g for 20 min at room temperature 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, 1 CaCl_2 , at 37°C for 6 h. Where indicated, erythrocytes were exposed to edelfosine (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 100 μl 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). 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".

Hemolysis

For the determination of hemolysis, the samples were centrifuged (10 min at 2000 rpm, room temperature) after incubation under the respective experimental conditions and 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.

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 μ M Fluo3/AM. The cells were incubated at 37°C for 30 min and washed once in Ringer solution containing 5 mM $CaCl_2$. The Fluo3/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.

Reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 100 μ l suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnelldorf, Germany) in PBS 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 in PBS. 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 and how edelfosine stimulates suicidal erythrocyte death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface.

In order to test whether edelfosine influences erythrocyte volume, forward scatter was determined utilizing flow cytometry following a 6 hours incubation in Ringer solution without or with edelfosine (1 – 5 μ M). As illustrated in Fig. 1, a 6 hours exposure to 1 and 2 μ M edelfosine did not significantly modify forward scatter, but 5 μ M edelfosine significantly decreased the average erythrocyte forward scatter.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. The erythrocytes were analysed following incubation for 6 hours in Ringer solution without or with edelfosine (1 - 5 μ M). As shown in Fig. 2, a 6

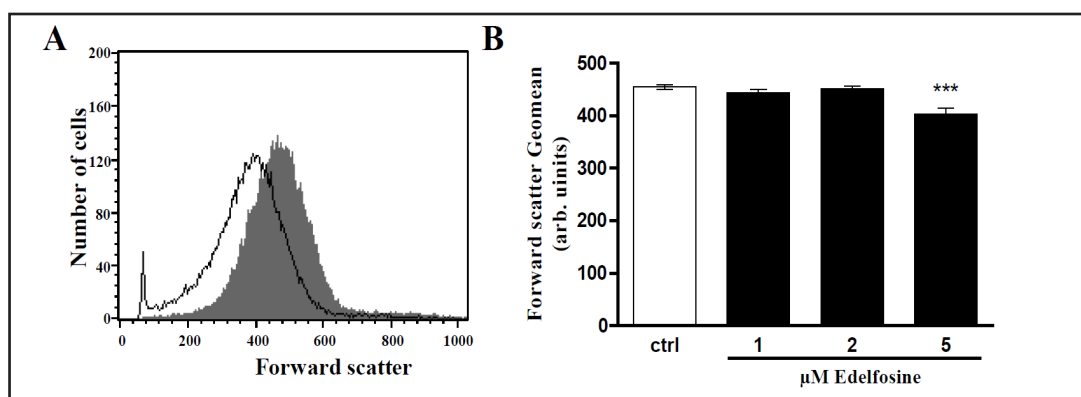


Fig. 1. Effect of edelfosine on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 6 hours to Ringer solution without (grey area) and with (black line) presence of 5 μ M edelfosine; B. Arithmetic means \pm SEM (*n* = 10) of the erythrocyte forward scatter (FSC) following incubation for 6 hours to Ringer solution without (white bar) or with (black bars) edelfosine (1 - 5 μ M). ***(*p*<0.001) indicate significant difference from the absence of edelfosine (ANOVA).

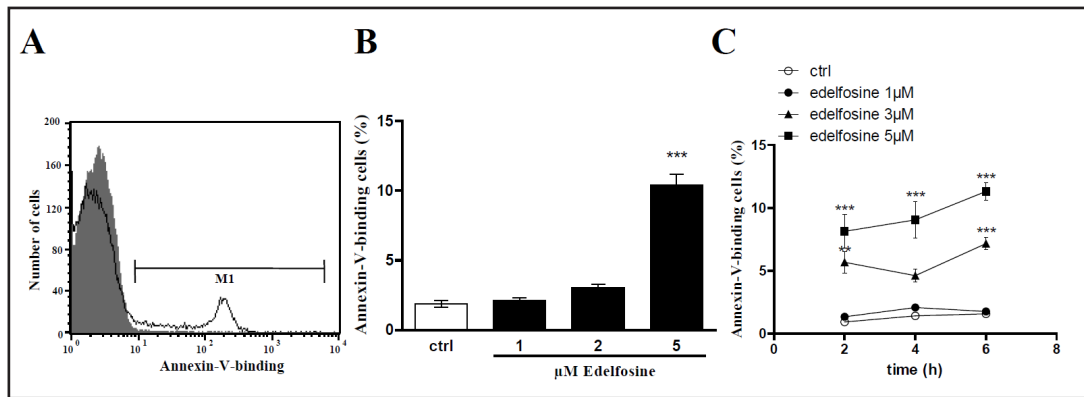


Fig. 2. Effect of edelfosine on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 6 hours to Ringer solution without (grey area) and with (black line) presence of 5 µM edelfosine; B. Arithmetic means \pm SEM (n = 10) of erythrocyte annexin-V-binding (black bars) following incubation for 6 hours to Ringer solution without or with presence of edelfosine (1 - 5 µM). C. Time course of the effect of different edelfosine concentrations on annexin-V-binding. ***($p < 0.001$) indicates significant difference from the absence of edelfosine (ANOVA).

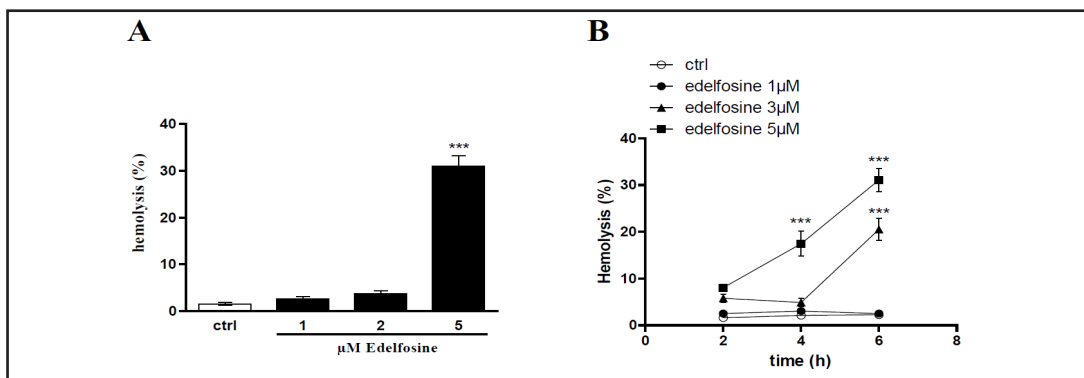


Fig. 3. Effect of edelfosine on hemolysis. Arithmetic means \pm SEM (n = 10) of the percentage hemolysed erythrocytes following incubation for 6 hours to Ringer solution without (white bar) or with (black bars) edelfosine (1 - 5 µM). B. Time course of the effect of different edelfosine concentrations on hemolysis. ***($p < 0.001$) indicates significant difference from the absence of edelfosine (ANOVA).

hours exposure to 1 and 2 µM edelfosine did not significantly modify annexin-V-binding, but 5 µM edelfosine significantly increased the percentage of phosphatidylserine exposing erythrocytes.

For quantification of hemolysis, the hemoglobin concentration in the supernatant was determined by photometry. As illustrated in Fig. 3, a 6 hours exposure to 1 and 2 µM edelfosine did not trigger significant hemolysis but 5 µM edelfosine significantly increased the percentage of hemolytic erythrocytes.

Fluo3-fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). As shown in Fig. 4, a 6 hours exposure to 1 and 2 µM edelfosine did not significantly modify Fluo3-fluorescence, but 5 µM edelfosine significantly increased the Fluo3-fluorescence.

In order to test whether edelfosine -induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca^{2+} , erythrocytes were incubated for 6 hours in the absence or presence of 5 µM edelfosine in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig 5, removal of extracellular Ca^{2+} significantly blunted the effect of edelfosine on annexin-V-binding. However, even in the absence extracellular Ca^{2+} edelfosine significantly increased the percentage of annexin-V-binding erythrocytes. Thus, the edelfosine-induced cell membrane scrambling was partially but not fully triggered by entry of extracellular Ca^{2+} .

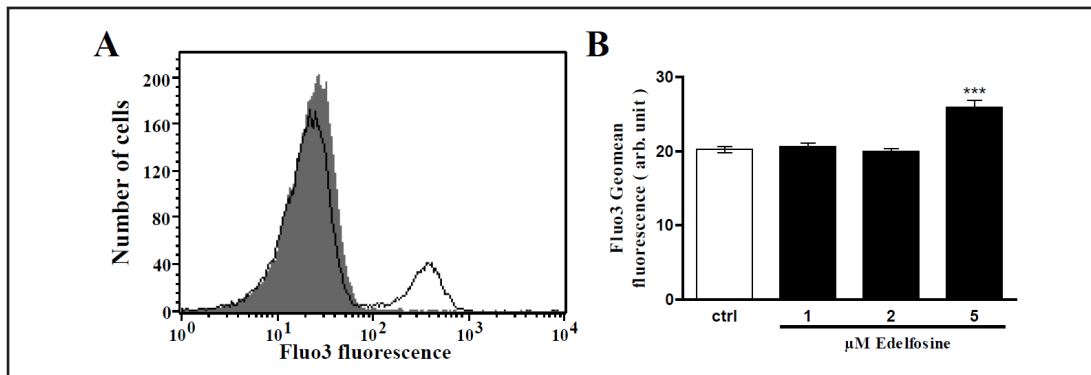


Fig. 4. Effect of edelfosine on erythrocyte Ca^{2+} activity. A. Original histogram of Fluo3-fluorescence in erythrocytes following exposure for 6 hours to Ringer solution without (grey area) and with (black line) presence of edelfosine ($5 \mu\text{M}$); B. Arithmetic means \pm SEM ($n = 10$) of the Fluo3-fluorescence (arbitrary units) in erythrocytes exposed for 6 hours to Ringer solution without (white bar) or with (black bars) edelfosine ($1 - 5 \mu\text{M}$). ***($p < 0.001$) indicates significant difference from the absence of edelfosine (ANOVA).

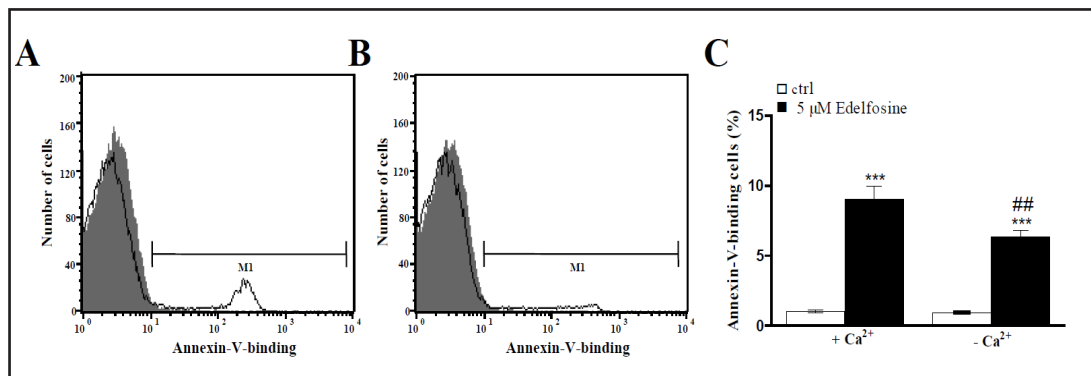


Fig. 5. Ca^{2+} sensitivity of edelfosine-induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 6 hours to Ringer solution without (grey area) and with (black line) presence of edelfosine ($5 \mu\text{M}$) in the presence (A) and absence (B) of extracellular Ca^{2+} ; C. Arithmetic means \pm SEM ($n = 10$) of annexin-V-binding of erythrocytes after a 6 hours treatment with Ringer solution without (white bars) or with (black bars) edelfosine ($5 \mu\text{M}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . ***($P < 0.001$) indicates significant difference from the absence of edelfosine, ##($p < 0.01$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Reactive oxygen species (ROS) was quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As a result, following a 6 hours incubation, the DCFDA fluorescence was similar in the absence of edelfosine (20.3 ± 0.6 $n = 6$) and in the presence of $1 \mu\text{M}$ (20.0 ± 0.6 $n = 6$), $2 \mu\text{M}$ (19.6 ± 1.3 $n = 6$) and $5 \mu\text{M}$ (18.5 ± 0.5 $n = 6$) edelfosine. Thus, edelfosine did not appreciably trigger oxidative stress.

Discussion

The present observations uncover a novel effect of edelfosine, i.e. the triggering of suicidal erythrocyte death or eryptosis. A six hours treatment of erythrocytes from healthy volunteers with $5 \mu\text{M}$ edelfosine is followed by erythrocyte shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentrations required for this effect are in the range of those encountered *in vivo* [17, 74].

The stimulation of phosphatidylserine translocation by edelfosine is at least in part due to triggering of Ca^{2+} entry from the extracellular space, as removal of extracellular Ca^{2+}

significantly blunted the edelfosine-induced cell membrane scrambling. An increase of $[Ca^{2+}]_i$ triggers cell membrane scrambling by activating an ill-defined scramblase [36]. An increase of $[Ca^{2+}]_i$ presumably further accounts for the erythrocyte shrinkage, as it leads to activation of Ca^{2+} sensitive K^+ channels with subsequent cell shrinkage due to K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [37].

Ca^{2+} entered presumably through Ca^{2+} permeable cation channels. Those channels could be opened by oxidative stress [36]. However, DCFDA fluorescence did not reveal a significant effect of edelfosine treatment on the abundance of reactive oxidant species. While this observation does not rule out effects of edelfosine on the redox state of the cation channels, other stimulators must be taken into consideration.

The edelfosine-induced eryptosis was paralleled by a stimulation of hemolysis. It is actually the purpose of eryptosis, to trigger removal of defective erythrocytes from circulating blood thus preceding hemolysis [36]. Hemolysis leads to release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules and thus occludes nephrons [75]. Eryptosis is particularly important for the clearance of *Plasmodium* infected erythrocytes in malaria. By imposing oxidative stress the pathogen activates the Ca^{2+} -permeable erythrocyte cation channels [36, 76]. The channels are more prone to be activated in several genetic erythrocyte disorders, such as sickle-cell trait, beta-thalassemia-trait, Hb-C-deficiency and G6PD-deficiency. The accelerated eryptosis and subsequent clearance of infected erythrocytes limits the parasitemia and thus confers partial protection against a severe clinical course of malaria [36, 77-79]. A similar relative protection is provided by iron deficiency [80], and treatment with lead [80], chlorpromazine [81] or NO synthase inhibitors [81], which all sensitize erythrocytes to the eryptotic effect of *Plasmodium* infection.

Accelerated eryptosis may lead, however, to anemia, as soon as the loss of erythrocytes cannot be compensated by similarly enhanced erythropoiesis [36]. Eryptosis may further lead to impairment of microcirculation [38, 82-86] due to adherence of phosphatidylserine exposing erythrocytes to the vascular wall [87], stimulation of blood clotting and triggering of thrombosis [82, 88, 89].

Conclusions

Edelfosine triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect paralleled by and in part due to stimulation of Ca^{2+} entry with increase of cytosolic Ca^{2+} activity.

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Disclosure Statement

The authors declare no conflict of interest.

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