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Original Paper

Stimulation of Suicidal Erythrocyte Death by PRIMA-1

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Key Words
Phosphatidylserine • PRIMA-1 • Calcium • Ceramide • Eryptosis

Abstract
Background: The anticarcinogenic drug PRIMA-1 (p53 reactivation and induction of massive apoptosis 1) induces suicidal death of tumor cells, an effect in large part attributed to the up-regulation of the proapoptotic transcription factor p53. Erythrocytes are lacking gene transcription but are nevertheless able to enter eryptosis, a suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$) and ceramide formation. The present study tested whether PRIMA-1 stimulates eryptosis. ***Methods:*** Phosphatidylserine exposure at the cell surface was estimated from annexin V binding, cell volume from forward scatter, $[Ca^{2+}]_i$ from Fluo3-fluorescence, ceramide abundance from binding of specific antibodies, and ROS formation from DCFDA fluorescence. ***Results:*** A 48 h exposure of human erythrocytes to PRIMA-1 (25 μM) significantly increased the percentage of annexin-V-binding cells without significantly influencing $[Ca^{2+}]_i$ or forward scatter. PRIMA-1 (100 μM) induced annexin-V-binding was not significantly blunted by removal of extracellular Ca^{2+} or by the caspase-3 inhibitor zVAD. PRIMA-1 (100 μM) further increased the ceramide abundance at the cell surface and ROS formation. ***Conclusions:*** PRIMA-1 stimulates phosphatidylserine translocation at the erythrocyte cell membrane, an effect at least partially due to up-regulation of ceramide abundance and ROS formation.

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Introduction

PRIMA-1 (p53 reactivation and induction of massive apoptosis 1 or APR-246), a widely used investigational drug for cancer therapy successfully tested in phase I/II clinical trials [1-3], triggers apoptosis of tumor cells and augments the tumor cell apoptosis following cytostatic treatment, radiation or hypoxia [1, 3-29]. The substance is at least partially effective by reactivating the proapoptotic transcription factor p53 [1, 3-5, 7, 8, 11-14, 16, 18-21, 23, 25-27, 30]. Moreover, PRIMA-1 up-regulates the related transcription factors p63 and p73 [2, 26]. PRIMA-1 may further stimulate autophagy [31]. In addition, PRIMA-1 derivatives have been shown to stimulate ceramide formation [32].

Erythrocytes lack nuclei and are unable to execute transcription factor dependent gene expression. Nevertheless, erythrocytes may enter suicidal cell death or eryptosis, which is characterized by cell shrinkage [33] and cell membrane scrambling with exposure of phosphatidylserine at the cell surface [34]. Signaling involved in the stimulation of eryptosis includes increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [34], formation of ceramide [35], oxidative stress [36], caspase activation [37-41], activation of casein kinase 1α [42, 43], Janus-activated kinase JAK3 [44], protein kinase C [45], or p38 kinase [46], as well as inhibition or knockout of AMP activated kinase AMPK [47], cGMP-dependent protein kinase [38], PAK2 kinase [48], sorafenib sensitive kinases [49] and sunitinib sensitive kinases [50].

The present study tested, whether PRIMA-1 is able to stimulate eryptosis. To this end, phosphatidylserine surface abundance, cell volume, $[Ca^{2+}]_i$, ceramide abundance, and ROS formation were determined in human erythrocytes from healthy individuals prior to and following treatment with PRIMA-1.

Materials and Methods

Ethics statement

All experiments in this manuscript have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The study is approved by the ethics committee of the University of Tübingen (184/2003 V).

Erythrocytes, solutions and chemicals

Fresh Lithium-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The blood was centrifuged at 120 rcf for 20 min at 23°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), 5 glucose, 1 $CaCl_2$; pH 7.4 at 37°C for 48 h. Where indicated, erythrocytes were exposed to PRIMA-1 (Sigma Aldrich, Schnelldorf, Germany) at the indicated concentrations, whereby 5 mg PRIMA-1 were solved in 270 µl H_2O to yield a 100 mM stock solution.

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 $CaCl_2$ 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. 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 (BD, 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 ceramide formation

To determine ceramide abundance, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 h at 37°C with 1µg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA) at a dilution of 1:10. After two washing steps with PBS-BSA, cells were stained for 30 min 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. Samples were then analyzed by flow cytometric analysis at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Quantification of reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 50 µl suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnelldorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 µM. 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 µl 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 ± 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 explored whether PRIMA-1 was capable to induce eryptosis, the suicidal erythrocyte death. The decisive hallmark of eryptosis is phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

In order to identify phosphatidylserine exposing erythrocytes, binding of labeled Annexin-V was determined utilizing flow cytometry. The measurements were performed following incubation of erythrocytes for 48 hours in Ringer solution without or with presence of PRIMA-1 (10 – 100 µM) prior to the measurements. As illustrated in Fig. 1, a 48 h exposure to PRIMA-1 enhanced the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 25 µM PRIMA-1. PRIMA-1 treatment thus resulted in erythrocyte cell membrane scrambling with subsequent translocation of phosphatidylserine to the cell surface. An extended dose response curve is provided in Fig.1C,D. Calculation of an EC50 from log[agonist] vs. normalized response (variable slope) utilizing Graphpad Prism software yielded a value of 3.5 mM. In a separate series, the percentage of Annexin-V binding erythrocytes was significantly ($p<0.05$) lower when 100 µM PRIMA-1 was added for 24 hours and the erythrocytes were subsequently exposed for additional 24 hours without PRIMA-1 (3.0 ± 0.5 %, n = 8) than when PRIMA-1 was added for 48 hours (5.0 ± 0.7 %, n = 8).

Alterations of cell volume were evidenced by alterations of forward scatter, which was again determined in flow cytometry. Forward scatter was quantified after incubation of the erythrocytes for 48 h in Ringer solution without or with PRIMA-1 (10 – 100 µM). As illustrated in Fig. 2, incubation of human erythrocytes in Ringer solution with PRIMA-1 tended to slightly decrease forward scatter, an effect, however, not reaching statistical significance.

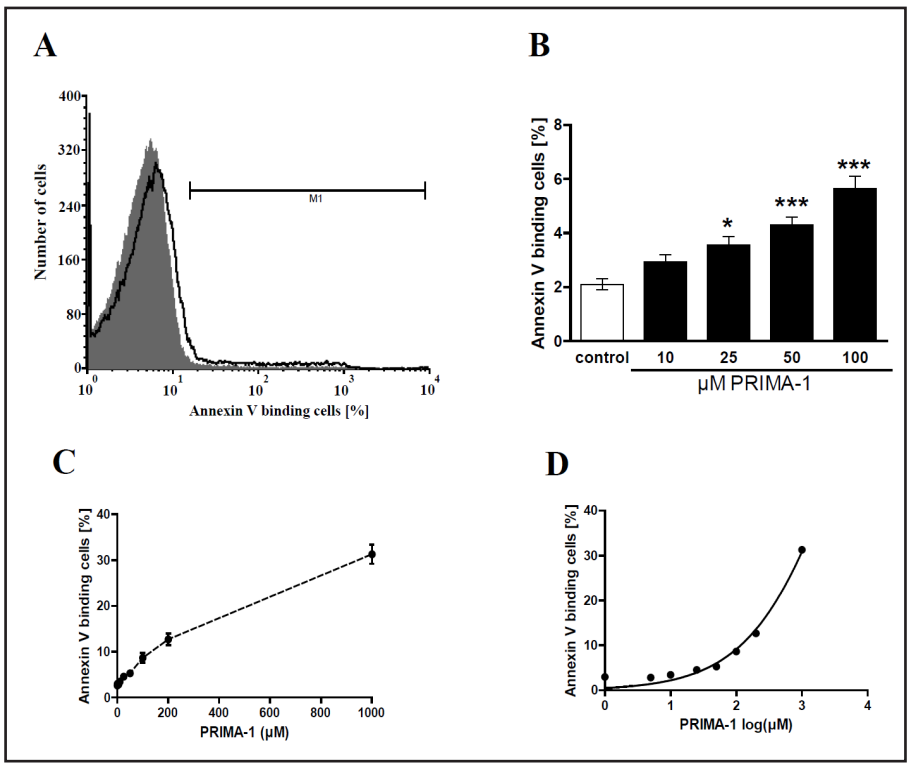

Fig. 1. Effect of PRIMA-1 on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes (M1) following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 100 μM PRIMA-1. B. Arithmetic means ± SEM of erythrocyte annexin-V-binding (n = 20) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of PRIMA-1 (10 - 100 μM). *(p<0.05), ***(p<0.001), indicates significant difference from the absence of PRIMA-1 (ANOVA). C,D. The percentage of erythrocytes binding annexin-V as a linear (C) and semilogarithmic (D) function of PRIMA-1 concentration (n=8).

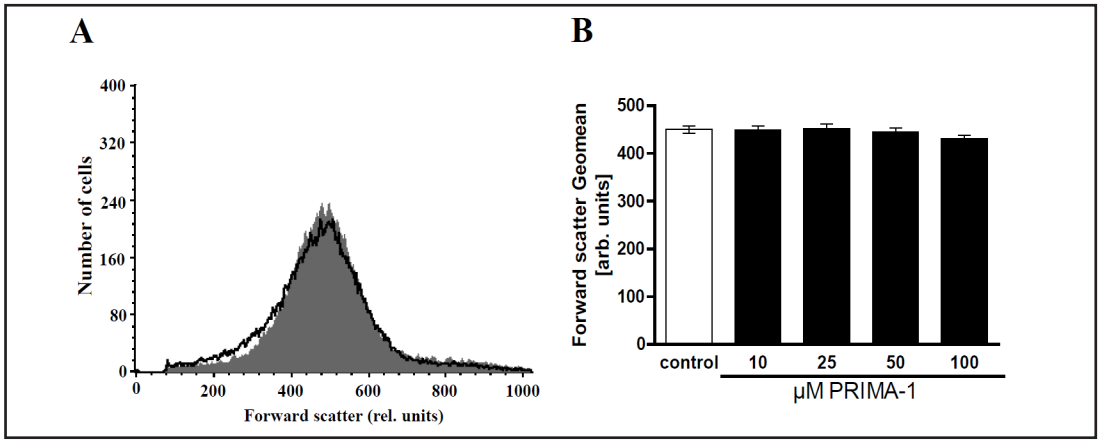

Fig. 2. Effect of PRIMA-1 on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 100 μM PRIMA-1. B. Arithmetic means ± SEM (n = 20) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of PRIMA-1 (10 – 100 μM). No significant differences were observed (ANOVA).

Alterations of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) were estimated utilizing Fluo3 fluorescence, which was again determined in flow cytometry. As shown in Fig. 3A,B, a 48 h exposure to PRIMA-1 (10 – 100 μM) did not significantly modify Fluo3 fluorescence, indicating that PRIMA-1 did not alter appreciably $[Ca^{2+}]_i$. Further experiments were performed to explore whether the PRIMA-1-induced cell membrane scrambling required entry of extracellular

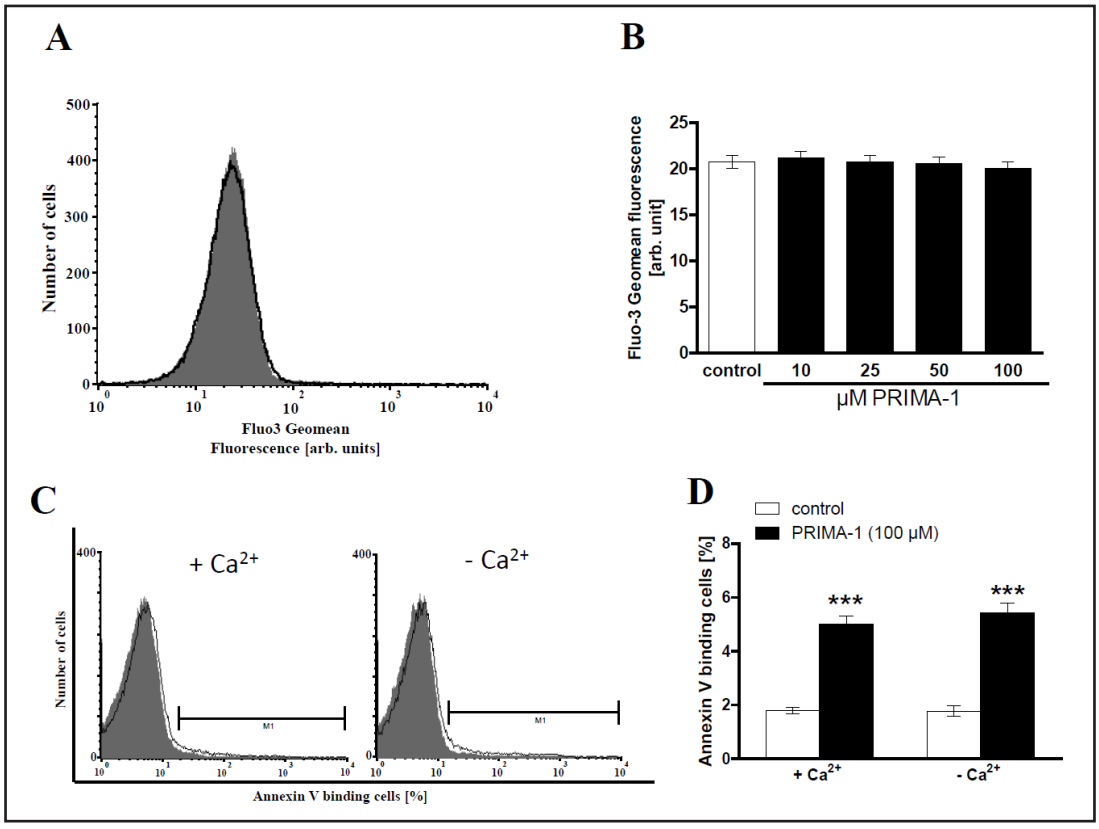

Fig. 3. Effect of PRIMA-1 on erythrocyte Ca^{2+} activity and Ca^{2+} dependence of PRIMA-1- induced phosphatidylserine exposure. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 100 µM PRIMA-1. B. Arithmetic means ± SEM (n = 20) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) presence of PRIMA-1 (10 – 100 µM). No significant differences were observed (ANOVA). C. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution in the prescense (left) or absence (right) of calcium, without (grey area) and with (black line) presence of 100 µM PRIMA-1. D. Arithmetic means ± SEM (n = 8) of erythrocyte forward scatter after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 100 µM PRIMA-1 in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of calcium. ***($p<0.001$) indicates significant difference from the absence of PRIMA-1.

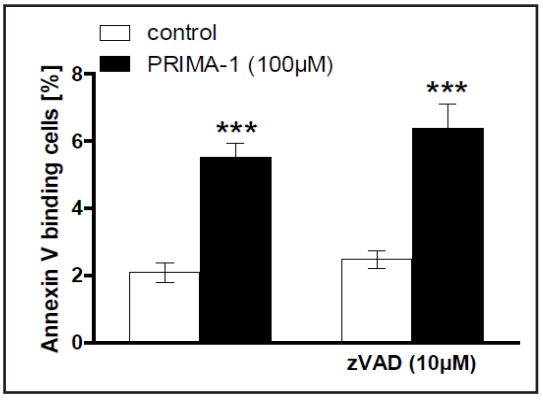

Fig. 4. PRIMA-1- induced annexin-V-bindingin presence of caspase inhibitor zVAD. Arithmetic means ± SEM (n = 8) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 100 µM PRIMA-1 in the absence (left bars) and presence (right bars) of caspase inhibitor zVAD (10 µM). *** ($p < 0.001$) indicates significant difference from the absence of PRIMA-1 (ANOVA).

Ca^{2+}. To this end, the erythrocytes were exposed for 48 h to 100 µM PRIMA-1 in the presence or nominal absence of extracellular Ca^{2+}. As shown in Fig. 3C, removal of extracellular Ca^{2+} did not significantly affect the increase of annexin-V-binding following PRIMA-1 treatment.

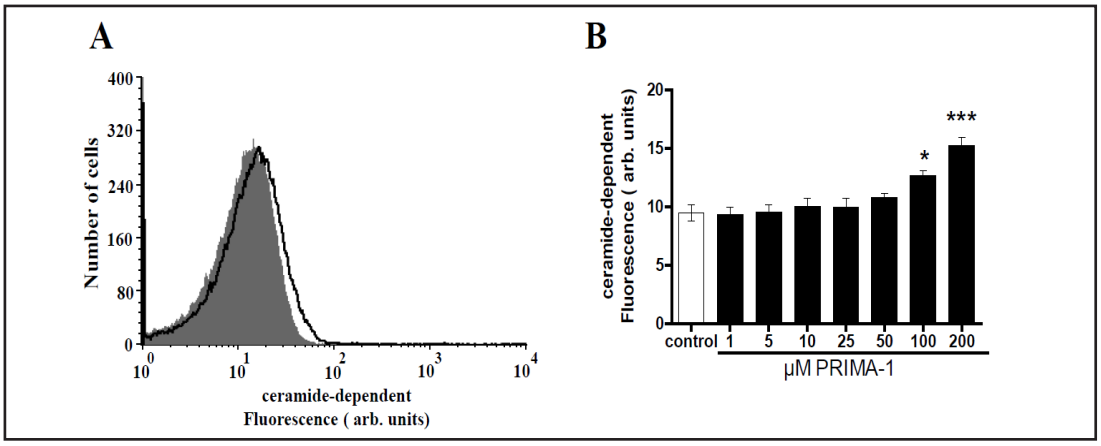

Fig. 5. Effect of PRIMA-1 on ceramide formation. A. Original histogram of ceramide surface abundance of erythrocytes following exposure for 48 h to Ringer solution without (grey shadow) and with (black line) presence of 100 μM PRIMA-1. B. Arithmetic means ± SEM (n= 8) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bars) 1-200 μM PRIMA-1. *(p<0.05), ***(p<0.001) indicates significant difference from the absence of PRIMA-1 (ANOVA).

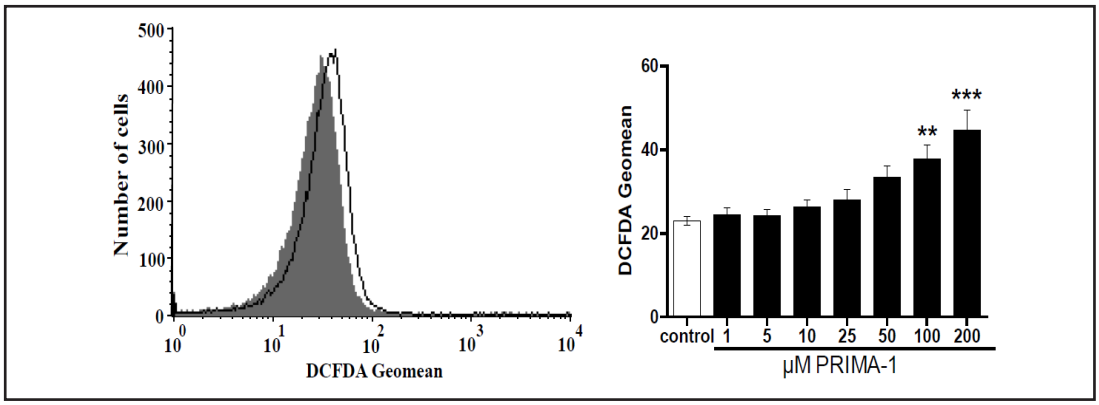

Fig. 6. Effect of PRIMA-1 on reactive oxygen species. A. Original histogram of 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 100 μM PRIMA-1. B. Arithmetic means ± SEM (n = 8) of erythrocyte DCFDA fluorescence following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) presence of PRIMA-1 (1 - 200 μM). **(p< 0.01), ***(p<0.001) indicates significant difference from the absence of PRIMA-1 (ANOVA).

Instead, PRIMA-1 significantly increased the percentage of annexin-V-binding erythrocytes even in the absence of extracellular Ca^{2+}. Thus, the effect of PRIMA-1 on phosphatidylserine translocation was mediated by a mechanism other than entry of extracellular Ca^{2+}.

A further series of experiments addressed the putative involvement of caspases. To this end, erythrocytes were exposed to 100 μM PRIMA-1 for 48 h either in the absence or presence of the pancaspase inhibitor zVAD (1 or 10 μM). As illustrated in Figure 4, zVAD did not significantly modify the effect of PRIMA-1 on annexin V binding.

As cell membrane scrambling could be triggered without requirement of increased $[Ca^{2+}]_i$ by ceramide, a further series of experiments explored, whether PRIMA-1-induced cell membrane scrambling was paralleled by formation of ceramide. To this end, the ceramide abundance at the erythrocyte surface was determined utilizing a specific anti-ceramide antibody. As shown in Fig. 5, a 48 h exposure of erythrocytes to PRIMA-1 increased the abundance of ceramide at the erythrocyte surface, an effect reaching statistical significance at 100μM.

In order to test whether PRIMA-1 enhances oxidative stress, reactive oxygen species (ROS) were determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA), As illustrated in fig.6, a 48 h treatment with PRIMA-1 increased DCFDA fluorescence, an effect reaching statistical significance at 100 μM.

Discussion

The present study reveals that exposure of human erythrocytes to high PRIMA-1 concentrations is followed by stimulation of cell membrane scrambling with phosphatidylserine translocation and increase of ceramide abundance at the erythrocyte surface. Phosphatidylserine exposure at the cell surface is a hallmark of eryptosis, the suicidal death of erythrocytes [34].

The cell membrane scrambling following PRIMA-1 treatment was not paralleled by an increase of $[Ca^{2+}]_i$. Moreover, removal ef extracellular Ca^{2+} did not significantly modify the stimulation of eryptosis by PRIMA-1. Instead, PRIMA-1 triggered phosphatidylserine exposure even in the absence of extracellular Ca^{2+}. Thus, PRIMA-1 was effective by mechanisms other than increase of $[Ca^{2+}]_i$. Moreover, the effect of PRIMA-1 was not significantly modified by the caspase inhibitor zVAD and was thus not dependent on caspase activation. Instead PRIMA-1 stimulated the formation of ceramide, an effect well known to stimulate eryptosis [34]. PRIMA-1 derivatives have most recently been shown to stimulate ceramide formation in tumor cells [32]. Moreover, PRIMA-1 induced oxidative stress, a well known stimulator of erythrocyte cell membrane scrambling [34].

PRIMA-1 tended to decrease cell volume, an effect, however, not reaching statistical significance. Thus, PRIMA-1 failed to significantly trigger the second hallmark of eryptosis. Moreover, PRIMA-1 apparently did not trigger membrane blebbing with formation of small particles, a further hall mark of eryptosis. This observation parallels the lack of PRIMA-1 effect on $[Ca^{2+}]_i$. Eryptotic cell shrinkage is usually caused by increase of $[Ca^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, hyperpolarization of the cell membrane, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [33]. The possibility must be kept in mind that PRIMA-1 triggers a programmed necrosis, a suicidal death distinct from eryptosis [51].

The PRIMA-1 concentration (25 μM) required for statistically significant stimulation of erythrocyte cell membrane scrambling was higher than those triggering apoptosis in tumor cells [12, 16]. PRIMA-1 tended to increase phosphatidylserine exposure at lower concentrations (10 μM), an effect, however, not reaching statistical significance. It must be kept in mind that erythrocytes may be sensitized to the effect of PRIMA-1 by other xenobiotics stimulating cell membrane scrambling [35, 49, 50, 52-82] or by diseases associated with enhanced cell membrane scrambling, such as sepsis, malaria, sickle cell disease, Wilson's disease, iron deficiency, malignancy, metabolic syndrome, diabetes, hepatic failure, renal insufficiency, hemolytic uremic syndrome, hyperphosphatemia and phosphate depletion [34, 83, 84].

The sensitization of erythrocytes for cell membrane scrambling by ceramide may, at least in theory, be relevant in malaria. The malaria pathogen *Plasmodium* triggers eryptosis by induction of oxidative stress, which activates several ion channels of the host cell membrane including Ca^{2+}-permeable erythrocyte cation channels [85, 86]. The Ca^{2+} entry through unselective cation channels triggers eryptosis with subsequent clearance of the infected erythrocytes from circulating blood [87]. Several genetic erythrocyte disorders including sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C and homozygous G6PD-deficiency enhance the susceptibility of erythrocytes for eryptosis and thus confer some protection against a severe course of malaria [34, 88-90]. Moreover, the clinical course of malaria is favourably influenced by clinical conditions with accelerated eryptosis, such as iron deficiency [91]. Eryptosis triggering xenobiotics shown to favourably influence the clinical course of malaria include lead [92], chlorpromazine [93] or NO synthase inhibitors [94]. Whether or not PRIMA-1 may influence the clinical course of malaria remains to be tested.

On the other hand, eryptosis may lead to anemia due to phagocytosis and subsequent removal of phosphatidylserine exposing erythrocytes. Clinically overt anemia is observed as soon as the rate of eryptosis exceeds the formation of new erythrocytes [34]. The binding of phosphatidylserine exposing erythrocytes to endothelial CXCL16/SR-PSO may in addition lead to adherance of eryptotic erythrocytes to the vascular wall [95]. Phosphatidylserine exposing erythrocytes may further stimulate blood clotting and thrombosis [96-98]. As a result phosphatidylserine exposing erythrocytes may impair microcirculation [35, 96, 99-102].

In conclusion, PRIMA-1 stimulates erythrocyte cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface, an effect at least partially due to stimulation of ceramide formation.

Disclosure Statement

The authors declare that they have no conflict of interest.

Acknowledgements

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