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**Original Paper**

# Triggering of Suicidal Erythrocyte Death by Psammaplin A

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**Key Words**
Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Ceramide • Calcium

**Abstract**
***Background/Aims:*** Psammaplin A, a natural product isolated from marine sponges, triggers apoptosis of tumor cells and is thus considered for the treatment of malignancy. In analogy to apoptosis of nucleated tumor cells, erythrocytes may enter eryptosis, a suicidal death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Cellular mechanisms stimulating eryptosis include increase of cytosolic $Ca^{2+}$ activity ($[Ca^{2+}]_i$), oxidative stress and ceramide. The present study explored, whether Psammaplin A induces eryptosis and to possibly shed some light on the underlying mechanisms. ***Methods:*** Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, cell volume was estimated from forward scatter, $[Ca^{2+}]_i$ determined utilizing Fluo3-fluorescence, the abundance of reactive oxygen species (ROS) quantified with DCFDA dependent fluorescence, and ceramide abundance at the erythrocyte surface detected with specific antibodies. ***Results:*** A 48 hours exposure of human erythrocytes to Psammaplin A (2-8 µg/ml) significantly decreased forward scatter and significantly increased the percentage of annexin-V-binding cells. Psammaplin A significantly increased Fluo3-fluorescence, the effect of Psammaplin A on annexin-V-binding and forward scatter was, however, not significantly blunted by removal of extracellular $Ca^{2+}$. Psammaplin A significantly increased DCFDA fluorescence and ceramide abundance. ***Conclusions:*** Psammaplin A triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect paralleled by increase of $[Ca^{2+}]_i$, induction of oxidative stress and enhanced appearance of ceramide.

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## Introduction

Psammaplin A, a symmetrical bromotyrosine-derived disulfide isolated from marine sponges [1-5], has previously been shown to trigger apoptosis of tumor cells [6-9] and is considered a potential drug for the treatment of malignancy [6-14]. Psammaplin A inhibits histone deacetylase and proved to be a powerful epigenetic modifier [3, 6, 10, 15-21]. In

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addition, the drug inhibits hepatitis C virus NS3 helicase and has thus been considered as antiviral drug [22]. Psammaplin A further inhibits chitinolytic enzymes and has antifungal and antibacterial potency [23-27].

Several substances triggering apoptosis of nucleated cells similarly trigger suicidal death of erythrocytes or eryptosis [28-70]. Eryptosis is characterized by cell shrinkage [71] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [59]. Mechanisms involved in the stimulation of eryptosis include increase of cytosolic $Ca^{2+}$ activity ($[Ca^{2+}]_i$) [59], ceramide [72], oxidative stress [59], energy depletion [59], caspases [59, 73, 74], and several kinases, such as casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, and p38 kinase [59]. Other kinases, such as AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase and sorafenib/sunitinib sensitive kinases counteract eryptosis [59].

The present study explored, whether Psammaplin A may trigger eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to Psammaplin A and phosphatidylserine translocation, cell volume, $[Ca^{2+}]_i$, oxidative stress, 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, 1 $CaCl_2$, at 37°C for 48 hours. Where indicated, erythrocytes were exposed for 48 hours to Psammaplin A (Santa Cruz Biotechnology, Texas, USA).

*Annexin-V-binding and forward scatter*

After incubation under the respective experimental condition, a 150 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 15 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 Psammaplin A treated erythrocytes.

*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 once in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer solution. Then, $Ca^{2+}$-dependent fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. Afterwards, the geomean of the $Ca^{2+}$ dependent fluorescence was determined.

*Reactive oxygen species (ROS)*

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 µl suspension of erythrocytes was washed in Ringer solution and stained with DCFDA (Sigma, Schnelldorf, Germany) at a final concentration of 10 µM. Erythrocytes were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 µl Ringer solution and ROS-dependent fluorescence intensity was measured in FL-1 at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD). Subsequently, the geomean of the DCFDA dependent fluorescence was determined.



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*Ceramide abundance*

To determine the ceramide abundance at the erythrocyte surface, a monoclonal antibody was used. After incubation, cells were stained for 1 h at 37°C with 1 µg/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/IgM (concentration 0.5 mg/ml) specific antibody (BD 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 in FL-1 at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Finally, the geomean of the ceramide dependent fluorescence was determined.

*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 aimed to possibly disclose an effect of Psammaplin A on eryptosis, the suicidal erythrocyte death, which is characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Forward scatter in flow cytometry was taken as measure of erythrocyte volume. The measurements were performed after a 48 hours incubation of the erythrocytes in Ringer solution without or with Psammaplin A (2 – 8 µg/ml). As shown in Fig. 1, Psammaplin A significantly decreased erythrocyte forward scatter at all Psammaplin A concentrations tested.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, which was quantified by flow cytometry. The measurements were again made after a 48 hours incubation of the erythrocytes in Ringer solution without or with Psammaplin A (2

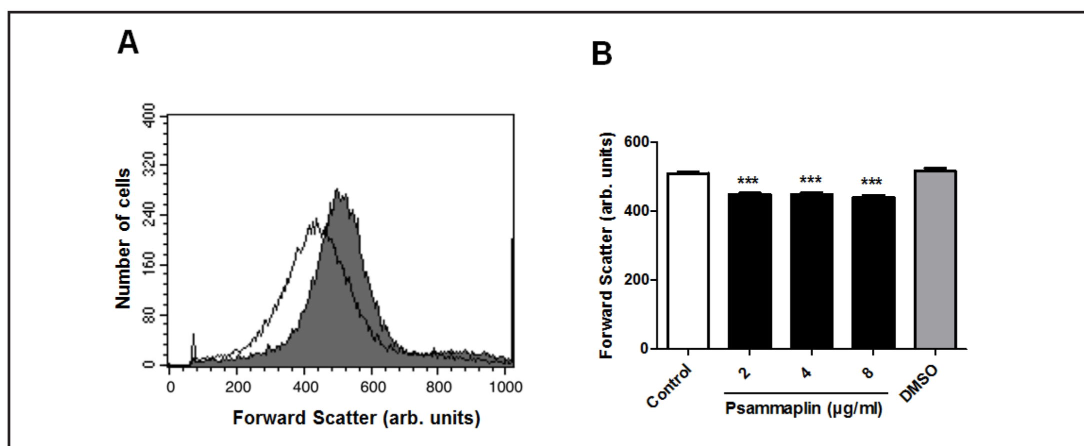


**Fig. 1.** Effect of Psammaplin A on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 8 µg/ml Psammaplin A. B. Arithmetic means ± SEM (n = 8) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Psammaplin A (2 - 8 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). ***($p<0.001$) indicates significant difference from the absence of Psammaplin A (ANOVA).



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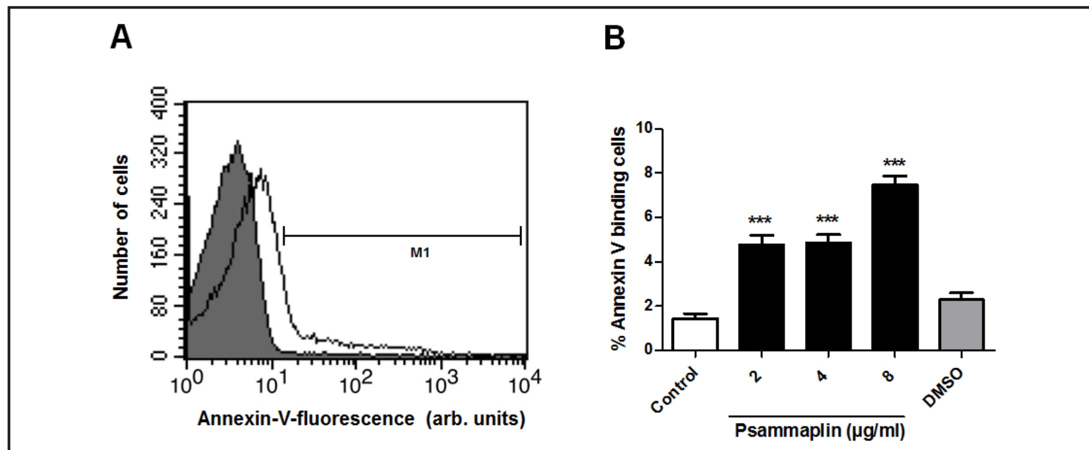


**Fig. 2.** Effect of Psammaplin A on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 8 µg/ml Psammaplin A. B. Arithmetic means ± SEM (n = 8) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Psammaplin A (2 - 8 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). ***(p<0.001) indicates significant difference from the absence of Psammaplin A (ANOVA).

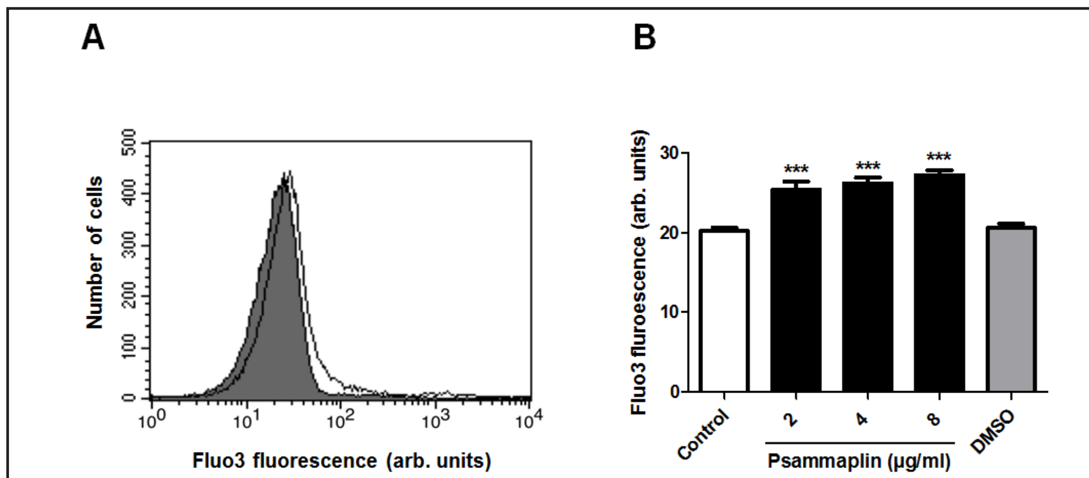


**Fig. 3.** Effect of Psammaplin A on Fluo3 fluorescenc. A. Original histogram of Fluo3 fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 8 µg/ml Psammaplin A. B. Arithmetic means ± SEM (n = 13) of erythrocyte Fluo3 fluorescence following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Psammaplin A (2 - 8 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). ***(p<0.001) indicates significant difference from the absence of Psammaplin A (ANOVA).

– 8 µg/ml). As illustrated in Fig. 2, a 48 hours exposure to Psammaplin A (2 – 8 µg/ml) significantly increased the percentage of phosphatidylserine exposing erythrocytes.

Cytosolic $Ca^{2+}$ activity ($[Ca^{2+}]_i$) was estimated utilizing Fluo3 fluorescence. The measurements were again made after a 48 hours incubation of the erythrocytes in Ringer solution without or with Psammaplin A (2 – 8 µg/ml). As apparent from Fig. 3, a 48 hours exposure to Psammaplin A (2 – 8 µg/ml) increased the Fluo3 fluorescence, an effect statistically significant at all Psammaplin A concentrations tested.

In order to test, whether the Psammaplin A-induced cell shrinkage and translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular $Ca^{2+}$, erythrocytes were incubated for 48 hours in the absence or presence of 8 µg/ml Psammaplin



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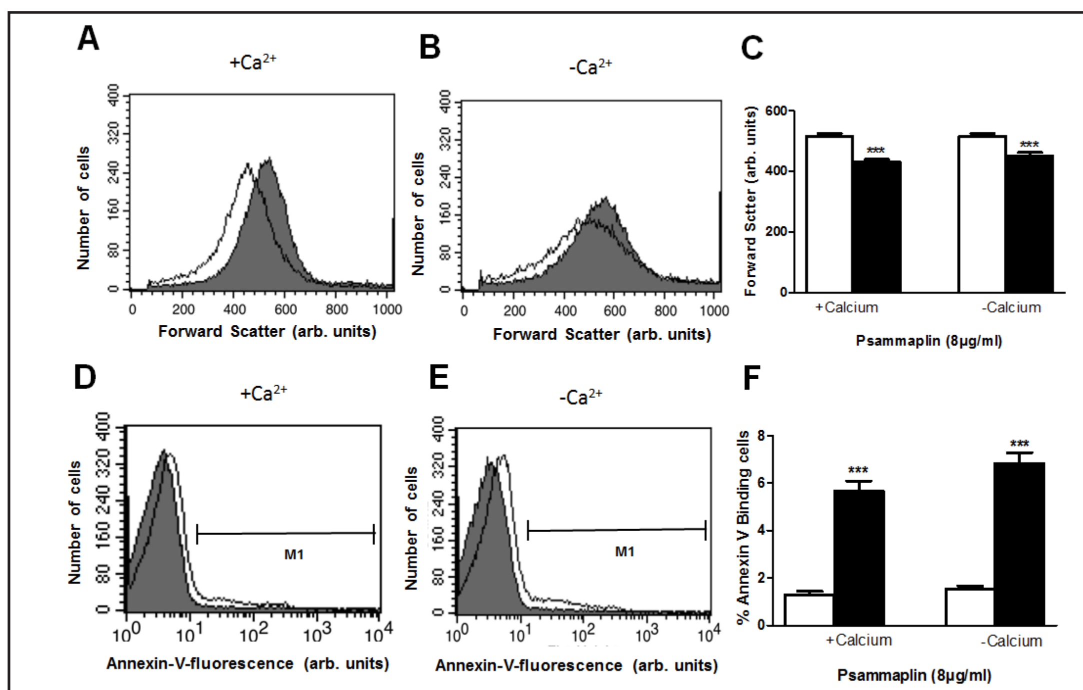


**Fig. 4.** $Ca^{2+}$ insensitivity of Psammaplin A-induced erythrocyte shrinkage and phosphatidylserine exposure. A,B. Original histograms of erythrocyte forward scatter following exposure for 48 hours to Ringer solution without (grey area) and with (black line) Psammaplin A (8 µg/ml) in the presence (A) and absence (B) of extracellular $Ca^{2+}$. C. Arithmetic means ± SEM (n = 13) of erythrocyte forward scatter after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Psammaplin A (8 µg/ml) in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of $Ca^{2+}$. ***($p<0.001$) indicates significant difference from the absence of Psammaplin A (ANOVA). D,E. Original histograms of annexin-V-exposing erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) Psammaplin A (8 µg/ml) in the presence (D) and absence (E) of extracellular $Ca^{2+}$. F. Arithmetic means ± SEM (n = 13) of the percentage of annexin-V-exposing erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Psammaplin A (8 µg/ml) in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of $Ca^{2+}$. ***($p<0.001$) indicates significant difference from the absence of Psammaplin A (ANOVA).

A in the presence or nominal absence of extracellular $Ca^{2+}$. As illustrated in Fig. 4A-C, removal of extracellular $Ca^{2+}$ did not significantly blunt the effect of Psammaplin A on forward scatter. Even in the absence of extracellular $Ca^{2+}$, Psammaplin A significantly decreased the erythrocyte forward scatter. Thus, Psammaplin A-induced cell shrinkage was in large part triggered by mechanisms other than entry of extracellular $Ca^{2+}$.

Similar observations were made with annexin-V-binding. Removal of extracellular $Ca^{2+}$ did not significantly blunt the effect of Psammaplin A on the percentage of annexin-V-binding erythrocytes and even in the absence of extracellular $Ca^{2+}$, Psammaplin A significantly increased the percentage of annexin-V-binding erythrocytes (Fig. 4D-F). Thus, Psammaplin A-induced cell membrane scrambling was in large part triggered by mechanisms other than entry of extracellular $Ca^{2+}$.

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). As illustrated in Fig. 5A,B, the DCFDA fluorescence was significantly increased following a 48 hours exposure to 8 µg/ml Psammaplin. Thus, Psammaplin A induced oxidative stress.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As a result, the ceramide abundance was significantly increased following a 48 hours exposure to 8 µg/ml Psammaplin (Fig. 5C,D).



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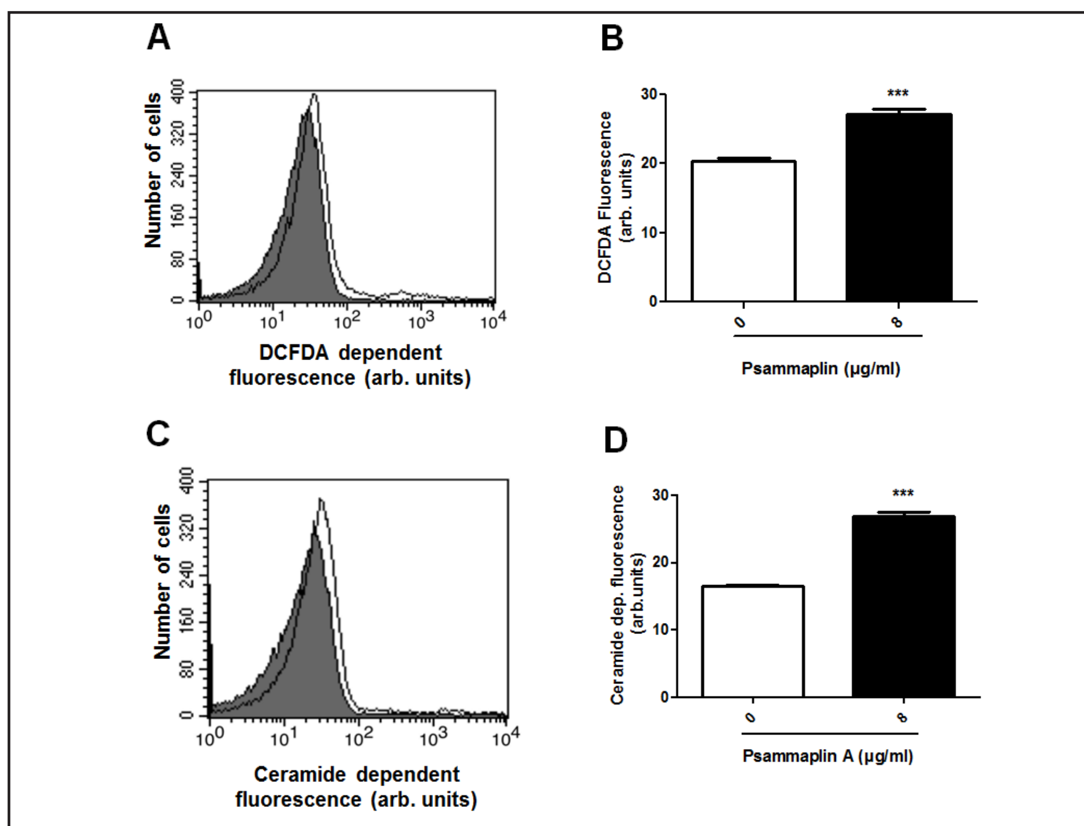


**Fig. 5.** Effect of Psammaplin A on DCFDA fluorescence and ceramide abundance. A. Original histogram of DCFDA fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 8 µg/ml Psammaplin A. B. Arithmetic means ± SEM (n = 9) of DCFDA fluorescence of erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) Psammaplin A (8 µg/ml). ***(p<0.001) indicates significant difference from the absence of Psammaplin A (unpaired *t* test). C. Original histogram of ceramide abundance at the surface of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 8 µg/ml Psammaplin A. D. Arithmetic means ± SEM (n = 5) of ceramide abundance at the surface of erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) Psammaplin A (8 µg/ml). ***(p<0.001) indicates significant difference from the absence of Psammaplin A (unpaired *t* test).

## Discussion

The present study revealed a novel effect of Psammaplin A, i.e. the stimulation of eryptosis, the suicidal erythrocyte death. A 48 hours treatment of eythrocytes with Psammaplin A was followed by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the erythrocyte surface. The concentration required to trigger eryptosis was in the range of plasma concentrations (up to 5.2 ± 0.8 µg/ml) encountered in Psammaplin treated rats [75]. The sensitivity to Psammaplin A is presumably enhanced in several clinical conditions with accelerated eryptosis, such as dehydration [76], hyperphosphatemia [77], chronic kidney disease (CKD) [78-81], hemolytic-uremic syndrome [82], diabetes [83], hepatic failure [84], malignancy [59], sepsis [85], sickle-cell disease [59], beta-thalassemia [59], Hb-C and G6PD-deficiency [59], as well as Wilson's disease [86].

The effect of Psammaplin A on cell membrane scrambling was paralleled by an increase of cytosolic $Ca^{2+}$ activity ($[Ca^{2+}]_i$). However, the Psammaplin A induced cell membrane scrambling was not appreciably blunted by removal of extracellular calcium and thus not dependent on $Ca^{2+}$

entry from the extracellular space. Accordingly, the effect of Psammaplin A on cell membrane scrambling was accomplished even in the absence of extracellular $Ca^{2+}$.

The effect of Psammaplin A on cell shrinkage was again seemingly independent from $Ca^{2+}$ entry from the extracellular space. Nevertheless, the Psammaplin A induced cell shrinkage could at least in part be due to increase of $[Ca^{2+}]_i$ with subsequent activation of $Ca^{2+}$ sensitive $K^+$ channels, $K^+$ exit, cell membrane hyperpolarization, $Cl^-$ exit and thus cellular loss of KCl with water.

Mechanisms possibly contributing to Psammaplin A induced eryptosis further include oxidative stress and ceramide, both well known stimulators of eryptosis [59].

The physiological significance of eryptosis is the removal of defective erythrocytes from circulating blood thus avoiding hemolysis [59] with release of hemoglobin, which could pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude the respective nephrons and thus cause renal failure [87]. Eryptosis may particularly lead to elimination of erythrocytes infected with the malaria pathogen *Plasmodium* [59]. It is tempting to speculate that Psammaplin A could accelerate the clearance of infected erythrocytes and thus favourably influence the clinical course of malaria.

Due to the rapid clearance of phosphatidylserine exposing erythrocytes from circulating blood, stimulation of eryptosis may lead, on the other hand, to anemia [59]. Excessive eryptosis may further compromize microcirculation [72, 88-92], as phosphatidylserine exposing erythrocytes adhere to the vascular wall [93], stimulate blood clotting and trigger thrombosis [88, 94, 95].

In conclusion, Psammaplin A triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect paralleled by increase of cytosolic $Ca^{2+}$ activity, oxidative stress and ceramide abundance.

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## Disclosure Statement

The authors state that they have no conflict of interest.

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