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Original Paper

Stimulation of Eryptosis, the Suicidal Erythrocyte Death, by Costunolide

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Key Words
Phosphatidylserine • Cell volume • Eryptosis • Calcium

Abstract
Background/Aims: The sesquiterpene lactone Costunolide is effective against various disorders including inflammation and malignancy. The substance is effective in part by triggering suicidal death or apoptosis of tumor cells. Mechanisms involved include altered function of transcription factors and mitochondria. Erythrocytes lack nuclei and mitochondria but are – in analogy to apoptosis of nucleated cells – able to enter suicidal erythrocyte death or eryptosis, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Triggers of eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), oxidative stress and ceramide. The present study explored, whether Costunolide induces eryptosis and, if so, to shed light on the mechanisms involved. ***Methods:*** Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, $[Ca^{2+}]_i$ from Fluo3-fluorescence, reactive oxygen species (ROS) formation from 2',7'-dichlorodihydrofluorescein (DCF)-dependent fluorescence, and ceramide abundance utilizing specific antibodies. ***Results:*** A 48 hours exposure of human erythrocytes to Costunolide (15 µg/ml) significantly enhanced the percentage of annexin-V-binding cells, significantly decreased forward scatter and significantly increased Fluo3-fluorescence, DCF-fluorescence, and ceramide abundance. The effect of Costunolide on annexin-V-binding was significantly blunted by removal of extracellular Ca^{2+}. ***Conclusion:*** Costunolide triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part due to Ca^{2+} entry and paralleled by oxidative stress and ceramide formation.

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Introduction

Costunolide, a sesquiterpene lactone isolated from Inula helenium (Compositae) [1, 2], has previously been shown to counteract inflammation [3-5], gastric ulcer [6], tumor growth [3, 7-11], metastasis [12, 13], angiogenesis [3], osteoclast differentiation [14], and fibrosis [3]. Costunolide is in part effective by decreasing vascular endothelial growth factor (VEGF) [3], interleukin (IL)-1β, IL-6, IL-17 [3], tumor necrosis factor (TNF)-α [3] and transforming growth factor (TGF-β) [3]. Costunolide induces oxidative stress [2, 11, 15-18] and fosters apoptosis of tumor cells [1, 2, 11, 16-24]. On the other hand costunolide protects against oxidative stress [25]. Signaling involved in the effects of Costulonide includes stimulation of mitogen-activated kinases [20, 26] as well as inhibition of the transcription factor NFκB [10, 13, 15] and the Wnt/β-Catenin Pathway [9]. The stimulation of apoptosis further involves mitochondria [19, 27].

Erythrocytes lack nuclei and mitochondria, key elements in the execution of apoptosis. but are nevertheless able to enter suicidal death of erythrocytes or eryptosis [28-30]. Hallmarks of eryptosis are cell shrinkage [31] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [1]. Cellular mechanisms involved in the triggering of eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [1], ceramide [32], caspases [28, 33, 34], G-protein Galphai2 [35], casein kinase 1α [1], Janus-activated kinase JAK3 [1], protein kinase C [1], and p38 kinase [1]. Eryptosis is suppressed by AMP activated kinase AMPK [1], cGMP-dependent protein kinase [1], mitogen and stress activated kinase MSK1/2 [36], and PAK2 kinase [1]. Stimulators of eryptosis include hyperosmotic shock [1], oxidative stress [1], energy depletion [1], radiation [37, 38], or exposure to a wide variety of substances [28, 36, 39-82]. Eryptosis could be inhibited by several small molecules [83-86]. Enhanced eryptosis is observed in a wide variety of clinical conditions including iron deficiency [1], dehydration [1], hyperphosphatemia [1], vitamin D excess [87], chronic kidney disease (CKD) [88-93], hemolytic-uremic syndrome [94], autoimmune hemolytic anemia [2], diabetes [96], hypertension and dyslipidemia [3], hepatic failure [98], malignancy [99-101], arteritis [102], systemic lupus erythematosus [103], sepsis [104, 105], malaria [28, 106, 107], sickle-cell disease [1], beta-thalassemia [1], Hb-C and G6PD-deficiency [1], Wilsons disease [108], as well as advanced age [4]. Eryptosis further increases following storage for transfusion [37, 38, 53, 109] and is enhanced in erythrocytes from newborns exposed to oxidative stress [28, 110].

The present study explored, whether Costunolide stimulates eryptosis. To this end human erythrocytes from healthy volunteers were exposed to Costunolide and phosphatidylserine surface abundance, cell volume, $[Ca^{2+}]_i$, ROS formation, 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 was approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 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 Costunolide (Sigma Aldrich, Hamburg, Germany). The incubation time was chosen in order to avoid failure to detect slow mechanisms triggering eryptosis.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 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). 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 Costunolide 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" [111].

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 µM Fluo-3/AM. The cells were incubated at 37°C for 30 min. 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 (DCF) diacetate. After incubation, a 100 µl suspension of erythrocytes was washed in Ringer solution and stained with DCFDA (Sigma, Schnelldorf, Germany) in Ringer solution containing DCF diacetate 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 at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. To this end, cells were stained for 1 hour at 37°C with 1 µM 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. 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 analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a control, secondary antibody alone was used.

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 addressed the potential effect of Costunolide on eryptosis, the suicidal erythrocyte death characterized by cell membrane scrambling with phosphatidylserine translocation to the cell surface.

Phosphatidylserine exposing erythrocytes were identified utilizing detection of annexin-V-binding by flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with Costunolide (5 – 15 µg/ml). As illustrated in Fig. 1, a 48 hours exposure to Costunolide increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 10 µg/ml Costunolide concentration.

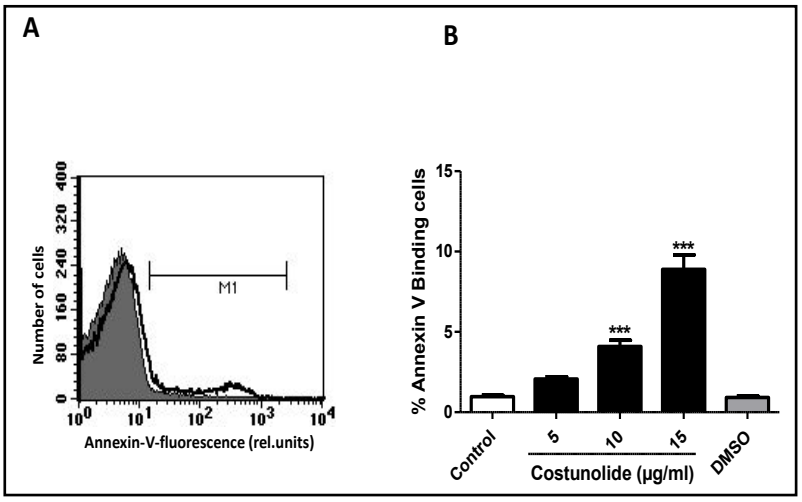

Fig. 1. Effect of Costunolide on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 15 µg/ml Costunolide. B. Arithmetic means ± SEM (n = 17) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Costunolide (5, 10, 15 µg/ml). For comparison, the effect of the solvent DMSO (grey bar). ***(p<0.001) indicates significant difference from the absence of Costunolide (ANOVA).

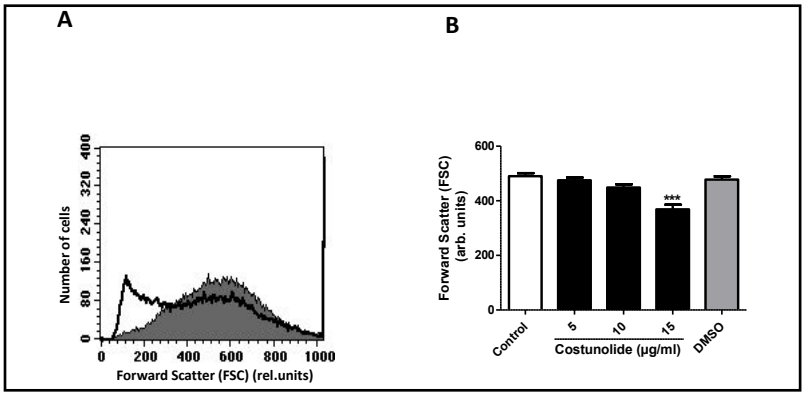

Fig. 2. Effect of Costunolide on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 15 µg/ml Costunolide. B. Arithmetic means ± SEM (n = 17) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Costunolide (5, 10, 15 µg/ml). ***(p<0.001) indicates significant difference from the absence of Costunolide (ANOVA).

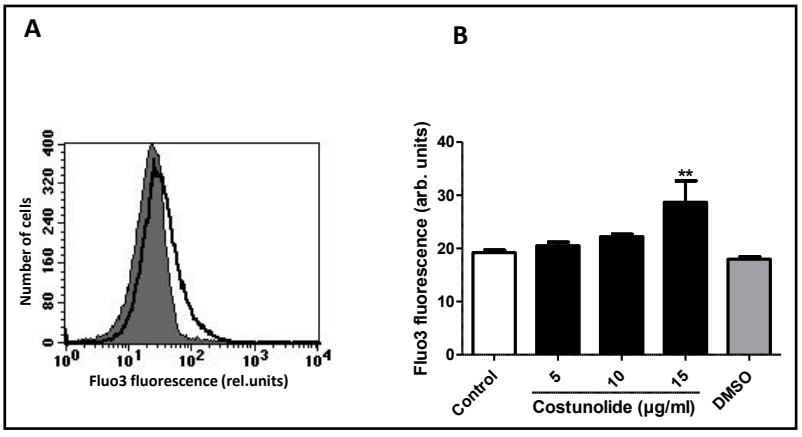

Fig. 3. Effect of Costunolide on cytosolic Ca^{2+} . A. Original histogram of Fluo3-fluorescence reflecting cytosolic Ca^{2+} activity of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 15 µg/ml Costunolide. B. Arithmetic means ± SEM (n = 13) of Fluo3-fluorescence reflecting cytosolic Ca^{2+} activity of erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Costunolide (5, 10, 15 µg/ml). For comparison, the effect of the solvent DMSO (grey bar). **(p<0.01) indicates significant difference from the absence of Costunolide (ANOVA).

Eryptosis is frequently paralleled by cell shrinkage. Thus erythrocyte volume was estimated from forward scatter determined with flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with Costunolide (5 – 15 µg/ml). As illustrated in Fig. 2, Costunolide (15 µg/ml) significantly decreased the average erythrocytes forward scatter. Moreover, Costunolide treatment was followed by an increase of the percentage of shrunken (<200) erythrocytes, an effect reaching statistical significance at 15 µg/ml Costunolide concentration. Again, erythrocyte treatment with costunolide also statistically increased the percentage of the swollen erythrocytes at 15 µg/ml concentration.

Fluo3-fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). As illustrated in Fig. 3, 48 hours incubation with 15 µg/ml Costunolide significantly increased the Fluo3-fluorescence.

In order to test whether the Costunolide-induced translocation of phosphatidylserine required entry of extracellular Ca^{2+}, erythrocytes were incubated for 48 hours in the absence or presence of 15 µg/ml Costunolide in the presence or nominal absence of extracellular Ca^{2+}. As illustrated in Fig. 4, removal of extracellular Ca^{2+} significantly blunted the effect of costunolide on annexin-V-binding. However, even in the absence of extracellular Ca^{2+}, Costunolide significantly increased the percentage of annexin-V-binding erythrocytes (Fig. 4). Thus, Costunolide-induced cell membrane scrambling was in part triggered by entry of extracellular Ca^{2+}.

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) were thus quantified utilizing 2′,7′-dichlorodihydrofluorescein (DCF) diacetate. As a result, a 48 hours incubation with 15 µg/ml Costunolide significantly increased the DCF-dependent fluorescence (Fig. 5).

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As illustrated in Fig. 6, a 48 hours exposure to 15 µg/ml Costunolide significantly increased the ceramide abundance.

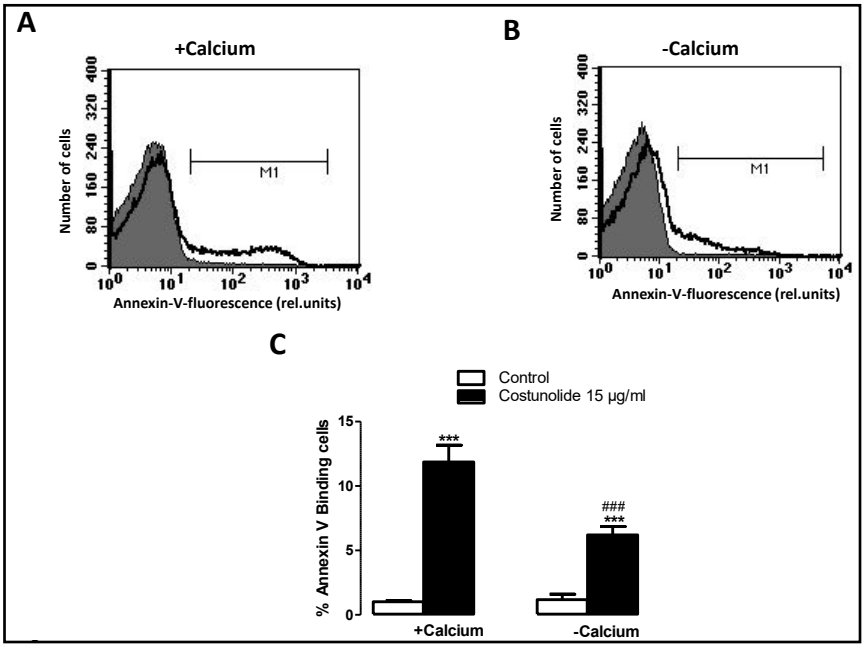

Fig. 4. Ca^{2+} sensitivity of Costunolide-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) Costunolide (15 µg/ml) in the presence (A) and absence (B) of extracellular Ca^{2+}. C. Arithmetic means ± SEM (n = 10) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with Costunolide (15 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 Costunolide, ###($p<0.001$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

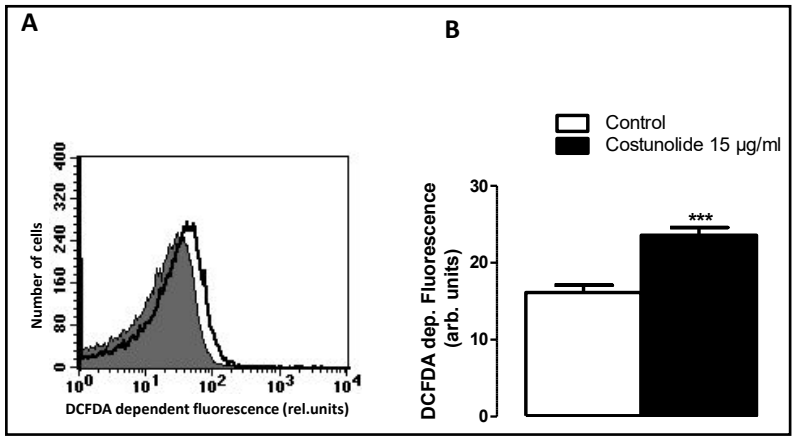

Fig. 5. Effect of Costunolide on oxidative stress. A. Original histogram of DCFDA fluorescence reflecting oxidative stress in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of Costunolide (15 µg/ml). B. Arithmetic means ± SEM (n = 10) of DCFDA fluorescence reflecting oxidative stress in erythrocytes following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) presence of Costunolide (15 µg/ml). ***(p<0.001) indicates significant difference from the absence of Costunolide (ANOVA).

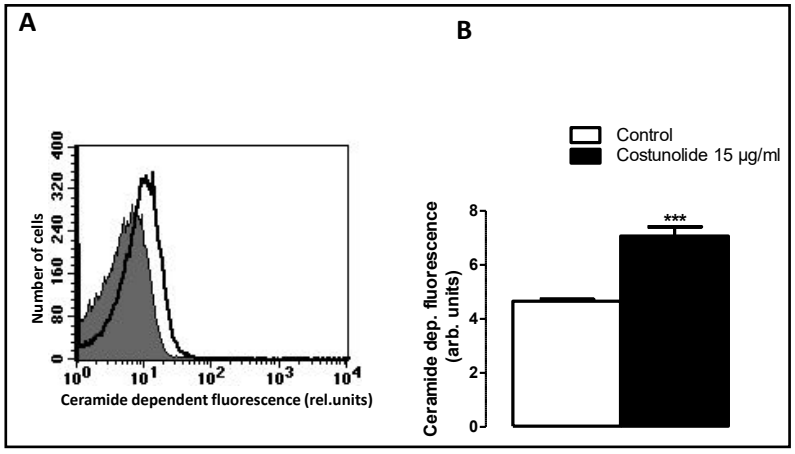

Fig. 6. Effect of Costunolide on ceramide abundance. A. Original histogram of ceramide abundance in erythrocytes following exposure for 48 hours to Ringer solution with solvent DMSO (grey area) and with presence of Costunolide (15 µg/ml) (black line). B. Arithmetic means ± SEM (n = 5) of ceramide abundance in erythrocytes following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) presence of Costunolide (15 µg/ml). ***(p<0.001) indicates significant difference from the absence of Costunolide (ANOVA).

Discussion

The present observations reveal that Costunolide triggers eryptosis, the suicidal erythrocyte death. Treatment of erythrocytes with Costunolide was followed by cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentration required for this effect was similar to those inducing apoptosis of nucleated cells, such as tumor cells [24]. The present observations were performed in erythrocytes drawn from healthy individuals. The sensitivity to Costunolide may be enhanced in clinical conditions with accelerated eryptosis.

The effect of Costunolide on cell membrane scrambling was paralleled by a significant increase of Fluo3-fluorescence reflecting cytosolic Ca^{2+} activity and was in large part dependent on Ca^{2+} entry from the extracellular space. Removal of extracellular Ca^{2+} significantly blunted the Costunolide induced eryptosis. Nevertheless, Costunolide triggered cell membrane scrambling even in the absence of extracellular Ca^{2+}, an observation pointing to the involvement of additional mechanisms.

Costunolide-induced cell membrane scrambling was paralleled by oxidative stress, a well known trigger of eryptosis [28]. Moreover, Costunolide increased the abundance of ceramide which is known to sensitize erythrocytes for the scrambling effect of Ca^{2+} [28].

Costunolide significantly decreased average forward scatter. Moreover, Costunolide significantly enhanced the percentage of shrunken erythrocytes. Costunolide-induced cell shrinkage could result from Ca^{2+} entry, activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [28]. The purpose of cell shrinkage is the avoidance of hemolysis leading to release of hemoglobin, which passes the renal glomerular filter and precipitates in the acidic lumen of renal tubules thus occluding the affected nephrons with subsequent renal failure [112]. Hemoglobin could further affect microcirculation [113].

Hemolysis and hemoglobin release could partially be prevented by eryptosis, since eryptotic erythrocytes are rapidly cleared from circulating blood and the timely triggering of eryptosis could lead to removal of defective erythrocytes prior to swelling and hemolysis of those cells [28]. Triggering of eryptosis may further lead to elimination of erythrocytes infected with the malaria pathogen *Plasmodium*, thus counteracting development of parasitemia [28].

On the other hand, the rapid clearance of phosphatidylserine exposing erythrocytes from circulating blood following stimulation of eryptosis may lead to anemia as soon as the loss of erythrocytes surpasses the formation of new erythrocytes by erythropoiesis [28]. Phosphatidylserine exposing erythrocytes may further adhere to endothelial cells of the vascular wall [114], stimulate blood clotting and trigger thrombosis [115-117], thus impairing microcirculation [32, 115, 118-121].

Conclusion

In conclusion, Costunolide triggers eryptosis with cell membrane scrambling, an effect paralleled by Ca^{2+} entry, oxidative stress and formation of ceramide.

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Disclosure Statement

All Authors confirm that they have no competing financial interests to disclose.

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