Cellular Physiology and Biochemistry

Cell Physiol Biochem 2015;37:131-142

DOI: 10.1159/000430339
Published online: August 17, 2015

Accepted: May 19, 2015

1421-9778/15/0371-0131$39.50/0

Original Paper

Triggering of Suicidal Erythrocyte Death Following Boswellic Acid Exposure

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Key Words
Phosphatidylserine • Cell volume • p38 kinase • Skepinone • SB203580 • Eryptosis

Abstract
Background/Aims: The antinflammatory natural product boswellic acid is effective against cancer at least in part by inducing tumor cell apoptosis. Similar to apoptosis of nucleated cells erythrocytes may enter eryptosis, a suicidal death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include oxidative stress, increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$), energy depletion, ceramide formation and p38 kinase activation. The present study tested, whether and how boswellic acid induces eryptosis. ***Methods:*** Phosphatidylserine exposure at the cell surface was estimated from annexin V binding, cell volume from forward scatter, hemolysis from hemoglobin release, $[Ca^{2+}]_i$ from Fluo3-fluorescence, ceramide abundance utilizing specific antibodies, reactive oxygen species (ROS) from 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, and cytosolic ATP concentration utilizing a luciferin–luciferase assay kit. ***Results:*** A 24 hours exposure of human erythrocytes to boswellic acid (5 µg/ml) significantly increased the percentage of annexin-V-binding cells (to 9.3 ±0.9 %) and significantly decreased forward scatter. Boswellic acid did not significantly modify $[Ca^{2+}]_i$, cytosolic ATP, ROS, or ceramide abundance. The effect of boswellic acid on annexin-V-binding was significantly blunted, but not abolished by p38 kinase inhibitors skepinone (2 µM) and SB203580 (2 µM). ***Conclusions:*** Boswellic acid stimulates cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part dependent on p38 protein kinase activity.

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Introduction

Boswellic acid, a component of *Boswellia serrata* extracts [1, 2] is a powerful modulator of the immune system [1, 2] and has been shown to counteract inflammation [1, 3-8] and malignancy [9-17]. The anticancer effect of boswellic acid and its derivatives is at least partially due to stimulation of suicidal tumor cell death or apoptosis [15, 18-28]. Mechanisms involved in boswellic acid induced apoptosis include downregulation of PI3K/Akt and ERK pathways [29-31], modulation of the Wnt/β-catenin signaling pathway [32], influence on p53/PUMA/Bax [33], topoisomerase I & II down-regulation [10, 11, 34], inhibition of mammalian target of rapamycin [35], down-regulation of NF-κB [36-39], altered gene expression [14, 39-44], mitochondrial depolarization [45], and caspase activation [23, 46-48].

Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage [49] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [50]. Cellular mechanisms triggering eryptosis include oxidative stress [50], Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [50], ceramide [51], energy depletion [50], activated caspases [50, 52, 53], stimulated activity of casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, and p38 kinase [50], as well as impaired activity of AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase and sorafenib/sunitinib sensitive kinases [50]. Eryptosis is further triggered by a wide variety of xenobiotics [50, 54-82].

The present study explored, whether and how boswellic acid triggers eryptosis. To this end, human erythrocytes from healthy volunteers were treated with boswellic acid and phosphatidylserine surface abundance and cell volume 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 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), 5 glucose, 1 $CaCl_2$; pH 7.4 at 37°C for 24 h. Where indicated, erythrocytes were exposed to boswellic acid (Sigma Aldrich, Hamburg, Germany), skepinone [83] or SB203580 (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 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 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".

Measurement of hemolysis

For the determination of hemolysis, the samples were centrifuged (3 min at 1600 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 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

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 hour at 37°C with 1 µg/ml 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. Subsequently, 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 then analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 100 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).

Determination of intracellular ATP concentration

For the determination of intracellular erythrocyte ATP, 90 µl of erythrocyte pellets were incubated for 24 h at 37°C in Ringer solution with or without boswellic acid (5 µg/ml). All subsequent manipulations were performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of $HClO_4$ (5%). After centrifugation, an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by addition of saturated $KHCO_3$ solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing a luciferin–luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol. ATP concentrations are expressed in mmol/l cytosol of erythrocytes.

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 the capacity of boswellic acid to stimulate suicidal erythrocyte death or eryptosis. Hallmarks of eryptosis include cell membrane scrambling with phosphatidylserine translocation to the cell surface. Phosphatidylserine at the erythrocyte surface was quantified utilizing annexin-V-binding, as determined by flow cytometry. The erythrocytes were analysed following incubation for 24 hours in Ringer solution without or with boswellic acid (0.5 - 5 µg/ml). As illustrated in Fig. 1B, a 24 hours exposure to 0.5 and 1 µg/ml did not significantly modify annexin-V-binding. However, incubation with 5 µg/ml boswellic acid significantly increased the percentage of annexin-V-binding erythrocytes.

In order to quantify hemolysis, the hemoglobin concentration in the supernatant was determined by photometry. As shown in Fig, 1B, a 24 hours incubation with boswellic acid (0.5 -5 µg/ml) did not significantly modify hemolysis.

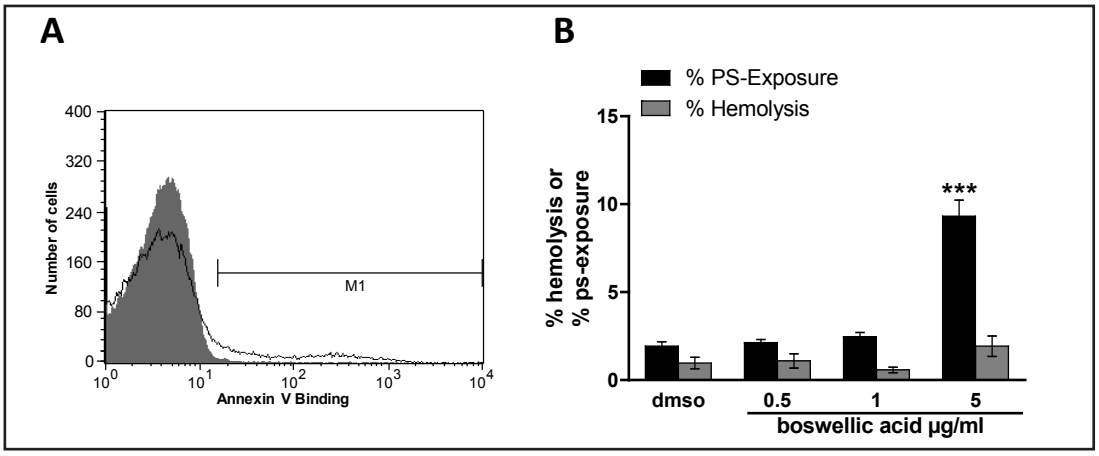

Fig. 1. Effect of boswellic acid on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of 5 µg/ml boswellic acid. B. Arithmetic means ± SEM (n = 8) of erythrocyte annexin-V-binding (black bars) following incubation for 24 hours to Ringer solution without or with presence of boswellic acid (0.5 - 5 µg/ml). For comparison, the effect of boswellic acid on hemolysis is shown (grey bars). *** ($p<0.001$) indicate significant difference from the absence of boswellic acid (ANOVA).

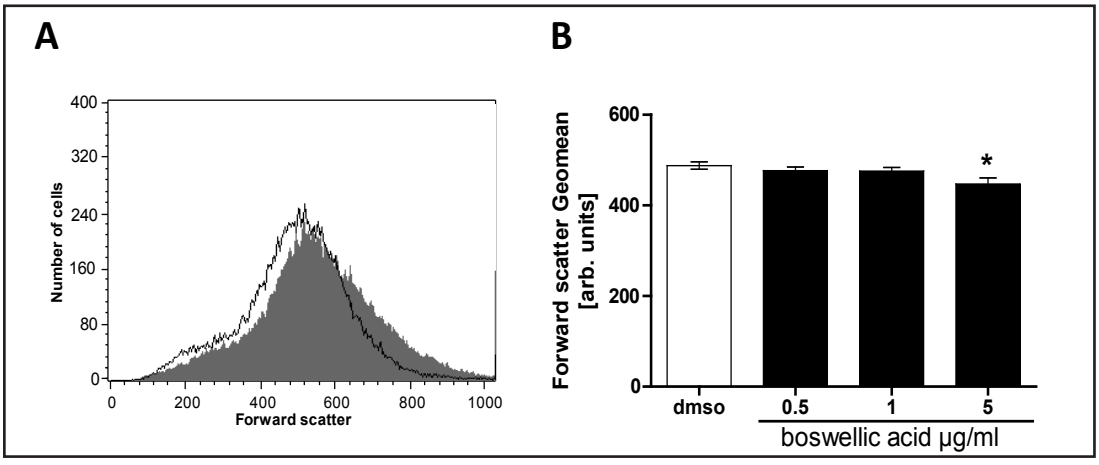

Fig. 2. Effect of boswellic acid on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 24 hours to Ringer solution without (grey area) and with (black line) presence of 5 µg/ml boswellic acid. B. Arithmetic means ± SEM (n = 8) of the erythrocyte forward scatter (FSC) following incubation for 24 hours to Ringer solution without (white bar) or with (black bars) boswellic acid (0.5 - 5 µg/ml). *($p<0.05$) indicate significant difference from the absence of boswellic acid (ANOVA).

The second hallmark of eryptosis is cell shrinkage. In order to estimate cell volume, forward scatter was determined utilizing flow cytometry following a 24 hours incubation in Ringer solution without or with boswellic acid (0.5 – 5 µg/ml). As shown in Fig, 2B, boswellic acid decreased erythrocyte forward scatter, an effect reaching statistical significance at 5 µg/ml boswellic acid concentration.

In order to determine whether boswellic acid modifies cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), Fluo3 fluorescence was taken as measure of $[Ca^{2+}]_i$. As a result, the Fluo3 fluorescence was similar following a 24 hours incubation without (23.3 ± 1.0 a.u., n = 8) and with 0.5 µg/ml (23.2 ± 1.0 a.u., n = 8) 1µg/ml (23.2 ± 1.0 a.u., n = 8) and 5 µg/ml (24.5 ± 0.9 a.u., n = 8) boswellic acid (0.5 – 10 µg/ml). Accordingly, boswellic acid did not significantly modify $[Ca^{2+}]_i$.

Stimulators of eryptosis in the absence of increased $[Ca^{2+}]_i$ include ceramide. Thus, specific antibodies were utilized to quantify ceramide abundance at the erythrocyte surface.

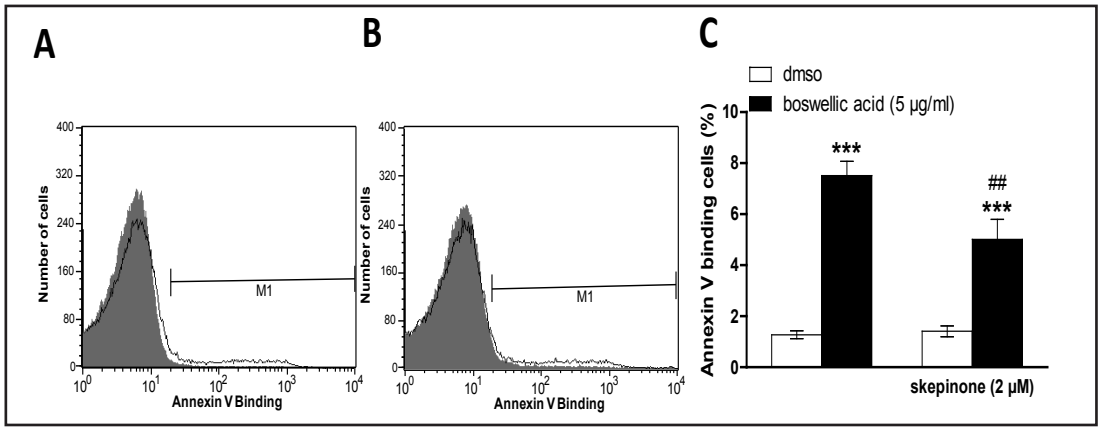

Fig. 3. Effect of boswellic acid on phosphatidylserine exposure in absence and presence of p38 kinase inhibitor skepinone. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution in the absence (A) and presence (B) of 5 µg/ml boswellic acid and with (grey area) and without (black line) presence of p38 kinase inhibitor skepinone (2 µM). C. Arithmetic means ± SEM of erythrocyte annexin-V-binding (n = 8) following incubation for 24 hours to Ringer solution without (white bars) or with (black bars) presence of boswellic acid (5 µg/ml) in the absence (left bars) and presence (right bars) of skepinone (2 µM). *** (p<0.001) indicate significant difference from the absence of boswellic acid, ## (p<0.01) indicate significant difference from the absence of skepinone (ANOVA).

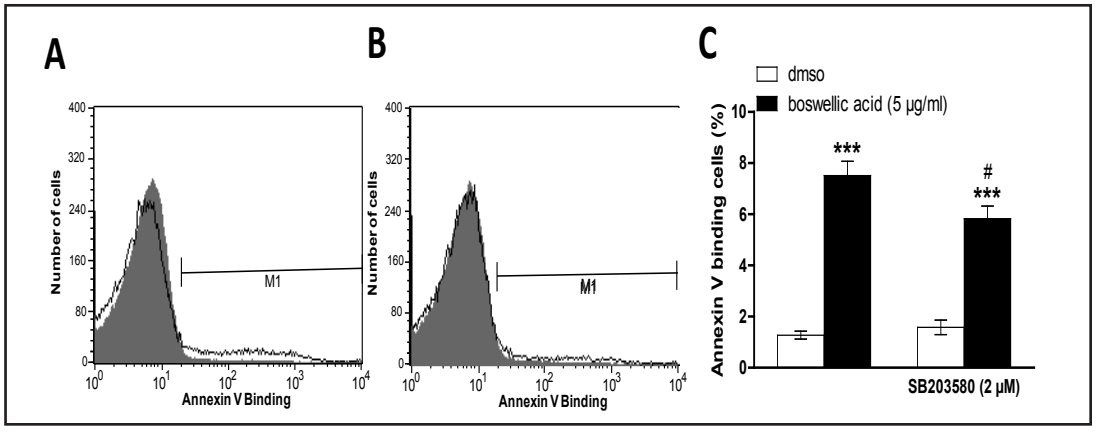

Fig. 4. Effect of boswellic acid on phosphatidylserine exposure in absence and presence of p38 kinase inhibitor SB203580. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 24 hours to Ringer solution in the absence (A) and presence (B) of 5 µg/ml boswellic acid and with (grey area) and without (black line) presence of p38 kinase inhibitor SB203580 (2 µM). C. Arithmetic means ± SEM of erythrocyte annexin-V-binding (n = 8) following incubation for 24 hours to Ringer solution without (white bars) or with (black bars) presence of boswellic acid (5 µg/ml) in the absence (left bars) and presence (right bars) of SB203580 (2 µM). *** (p<0.001) indicate significant difference from the absence of boswellic acid, # (p<0.05) indicate significant difference from the absence of SB203580 (ANOVA).

As a result, the ceramide abundance was similar following a 24 hours incubation in the absence (16.5 ± 1.9 a.u., n = 8) and presence (15.6 ± 1.7 a.u., n = 8) of 5 µg/ml boswellic acid.

Triggers of eryptosis include oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). As a result, the DCFDA fluorescence was similar following a 24 hours incubation in the absence (13.1 ± 1.2 a.u., n = 8) and presence (12.0 ± 1.1 a.u., n = 8) of 5 µg/ml boswellic acid.

Eryptosis is further stimulated by energy depletion. A luciferin–luciferase assay was employed to determine the cytosolic ATP level. As a result, the cytosolic ATP concentration

was similar following a 24 hours incubation in the absence (2.3 ± 0.4 mM, n = 8) and presence (1.9 ± 0.2 mM, n = 8) of 5 μg/ml boswellic acid.

Additional experiments explored whether boswellic acid-induced cell membrane scrambling was dependent on activation of p38 kinase. To this end, erythrocytes were exposed for 24 hours to 10 μg/ml boswellic acid in the absence or presence of the selective [83] p38 kinase inhibitor skepinone (2 μM) or selective [84] p38 kinase inhibitor SB203580 (2 μM). As a result, the effect of boswellic acid on annexin-V-binding was significantly blunted in the presence of p38 kinase inhibitors skepinone (Fig. 3) and SB203580 (Fig. 4).

Discussion

The present observations reveal a novel effect of boswellic acid, i.e. the stimulation of suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to boswellic acid is followed by the appearance of the two hallmarks of eryptosis, i.e. cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The boswellic acid concentration (5 μg/ml) required for stimulation of erythrocyte cell membrane scrambling was within the range of concentrations observed *in vivo* [85] and those required for stimulation of tumor cell apoptosis *in vitro* [25].

The effect of boswellic acid on cell membrane scrambling and cell shrinkage was apparently not due to increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). An increase of $[Ca^{2+}]_i$ accounts for the stimulation of cell membrane scrambling following treatment with a wide variety of xenobiotics [50]. An increase of $[Ca^{2+}]_i$ further activates 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 [49]. Even though the observations do not exclude minor alterations of $[Ca^{2+}]_i$, the strong effect of boswellic acid on cell membrane scrambling would require sizable increases of $[Ca^{2+}]_i$ [50].

The stimulation of cell membrane scrambling was further not paralleled by appreciable increases of reactive oxygen species (ROS), energy depletion or ceramide abundance at the cell surface. ROS, energy depletion and ceramide are well known triggers of eryptosis [50].

The boswellic acid induced eryptosis was significantly blunted by p38 kinase inhibitors skepinone and SB203580. Activation of p38 protein kinase has previously been shown to participate in the signaling of eryptosis [50].

Stimulation of eryptosis may be beneficial, as it allows disposal of defective erythrocytes prior to hemolysis [50]. Eryptosis thus prevents release of hemoglobin, which would be filtered in renal glomerula, precipitate in the acidic lumen of renal tubules and thus occlude nephrons [86]. Removal of eryptotic cells may further favourably influence the clinical course of malaria [87]. The malaria pathogen *Plasmodium* imposes oxidative stress on the infected host erythrocyte thus activating several host cell ion channels including Ca^{2+}-permeable erythrocyte cation channels [50, 88]. The stimulation of eryptosis by Ca^{2+} entry through those channels leads to subsequent clearance of infected erythrocytes from circulating blood [87]. By increasing the erythrocyte susceptibility to triggers of eryptosis, sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency protect against a severe course of malaria [50, 89-91]. Iron deficiency [92], and treatment with lead [92], chlorpromazine [93] or NO synthase inhibitors [93] similarly counteract parasitemia by increasing the erythrocyte susceptibility to triggers of eryptosis. It is tempting to speculate that boswellic acid may similarly foster eryptosis in *plasmodium* infected erythrocytes.

On the other hand, boswellic acid may aggravate anaema in clinical conditions associated with enhanced eryptosis, such as dehydration [70], hyperphosphatemia [81] chronic kidney disease (CKD) [59, 94-96], Hemolytic-uremic syndrome [97], diabetes [98], liver failure [99], malignancy [100], sepsis [101] and Wilsons disease [102]. Enhanced eryptosis with clearance of phosphatidylserine exposing erythrocytes from circulating blood leads to anemia, as soon as the loss of erythrocytes outcasts the generation of new erythrocytes [50]. Eryptotic erythrocytes may further adhere to the vascular wall [103], trigger blood clotting

and elicit thrombosis [104-106]. Eryptotic erythrocytes may thus impair microcirculation [51, 104, 107-110].

In conclusion, boswellic acid triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect paralleled by and in part due to activation of p38 protein kinase.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch. The study was supported by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tuebingen University.

Disclosure Statement

The authors declare no conflict of interest.

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