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Original Paper

Stimulating Effect of Sclareol on Suicidal Death of Human Erythrocytes

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Key Words
Phosphatidylserine • Cell volume • Eryptosis • Skepinone • D4476 • Calcium • Sclareol

Abstract
Background/Aims: The diterpene alcohol Sclareol has been proposed for the treatment of malignancy. In analogy to apoptosis of nucleated cells, erythrocytes may enter eryptosis, a suicidal cell death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Cellular mechanisms involved in the triggering of eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), oxidative stress, ceramide, p38 kinase and casein kinase 1α. The present study explored, whether Sclareol induces eryptosis and, if so, shed light on the mechanisms involved. ***Methods:*** Phosphatidylserine abundance at the erythrocyte surface was estimated from annexin-V-binding, cell volume from forward scatter, $[Ca^{2+}]_i$ from Fluo3-fluorescence, abundance of reactive oxygen species (ROS) from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA)-dependent fluorescence, and ceramide abundance at the erythrocyte surface utilizing specific antibodies. Hemolysis was estimated from haemoglobin concentration in the supernatant. ***Results:*** A 48 hours exposure of human erythrocytes to Sclareol (≥ 50 μM) significantly increased the percentage of annexin-V-binding cells without significantly modifying the average forward scatter, DCF-fluorescence or ceramide abundance. Sclareol (≥ 50 μM) further triggered hemolysis. Sclareol (100 μM) significantly increased Fluo3-fluorescence, but the effect of Sclareol on annexin-V-binding was not significantly blunted by removal of extracellular Ca^{2+}. Instead, the effect of Sclareol on annexin-V-binding was significantly blunted in the presence of p38 kinase inhibitor skepinone (2 μM) and in the presence of casein kinase 1α inhibitor D4476 (10 μM). ***Conclusions:*** Sclareol triggers phospholipid scrambling of the erythrocyte cell membrane, an effect in part due to activation of p38 kinase and casein kinase 1α.

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Introduction

Plant-derived terpenoids [1] such as the diterpene alcohol Sclareol [2] are effective against cancer [1, 3-13], inflammation [1, 14, 15], and infection [16-18]. Sclareol further influences brain function [19-25]. The effect of Sclareol against cancer is partially due to stimulation of tumor cell apoptosis [3-5, 7, 12, 13, 26]. Sclareol is incorporated into phospholipid model membranes [27] and has been shown to suppress the formation of nitric oxide [15] and prostaglandin E2 [15].

In analogy to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal erythrocyte death characterized by cell shrinkage [28] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [29]. Cellular mechanisms accomplishing eryptosis include Ca^{2+} entry with increase of cytotosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [29], ceramide [30], caspases [29, 31, 32], as well as activation of casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, and/or p38 kinase [29]. Eryptosis is further triggered by pharmacological inhibition or genetic knockout of AMP activated kinase, cGMP-dependent protein kinase, and PAK2 kinase [29], by oxidative stress [29], energy depletion [29] and diverse xenobiotics [29, 33-64]. Eryptosis is enhanced in several clinical conditions, such as dehydration [65], hyperphosphatemia [66], chronic kidney disease (CKD) [67-70], hemolytic-uremic syndrome [71], diabetes [72], hepatic failure [73], malignancy [29], sepsis [74], sickle-cell disease [29], beta-thalassemia [29], Hb-C and G6PD-deficiency [29], as well as Wilsons disease [75].

The present study analyzed Sclareol sensitivity of eryptosis. Human erythrocytes drawn from healthy volunteers were treated with Sclareol and phosphatidylserine surface abundance, cell volume, $[Ca^{2+}]_i$, reactive oxygen species (ROS), and ceramide abundance determined by flow cytometry. Moreover, the effect of Sclareol on cell membrane scrambling was quantified in the presence or absence of extracellular Ca^{2+}, of p38 kinase inhibitor skepinone and of casein kinase 1α inhibitor D4476.

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; pH 7.4), 5 glucose, 1 $CaCl_2$, at 37°C for 48 hours. Where indicated, erythrocytes were exposed for 48 hours to Sclareol (Sigma Aldrich, Hamburg, Germany). To test for an involvement of p38 kinase or casein kinase 1α, erythrocytes were exposed for 48 hours to a combination of Sclareol and p38 kinase inhibitor skepinone [76] or casein kinase 1α inhibitor D4476 (Tocris bioscience, Bristol, UK), respectively.

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 Sclareol treated erythrocytes.

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

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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 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 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 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 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. 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.

Hemolysis

For the determination of hemolysis, erythrocyte suspensions were centrifuged 10 min at 2000 rpm RT and the supernatants harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatants was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

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 Sclareol stimulates eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and by phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with Sclareol (10 - 100 µM). As illustrated in Fig. 1, a 48 hours exposure to Sclareol increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 100 µM Sclareol.

Erythrocyte volume was estimated from forward scatter which was determined utilizing flow cytometry. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with Sclareol (10 – 100 µM). As shown in Fig. 2, Sclareol did not significantly modify the average erythrocyte forward scatter, but at a concentration of 100 µM significantly decreased the percentage of large erythrocytes (>800).

As shown in Fig. 3, Sclareol exposure was further followed by hemolysis, an effect reaching statistical significance at 50 µM Sclareol.

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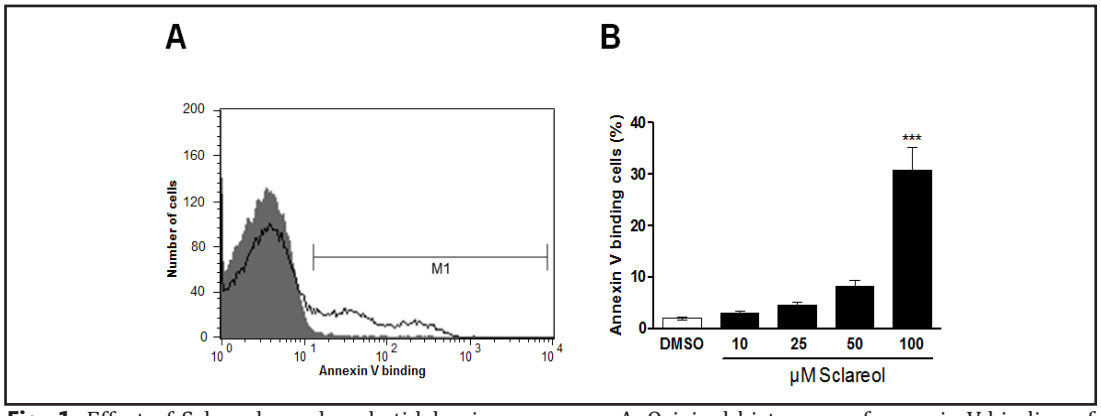

Fig. 1. Effect of Sclareol on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 100 µM Sclareol. B. Arithmetic means ± SEM (n = 10) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Sclareol (10 – 100 µM). ***(p<0.001) indicates significant difference from the absence of Sclareol (ANOVA).

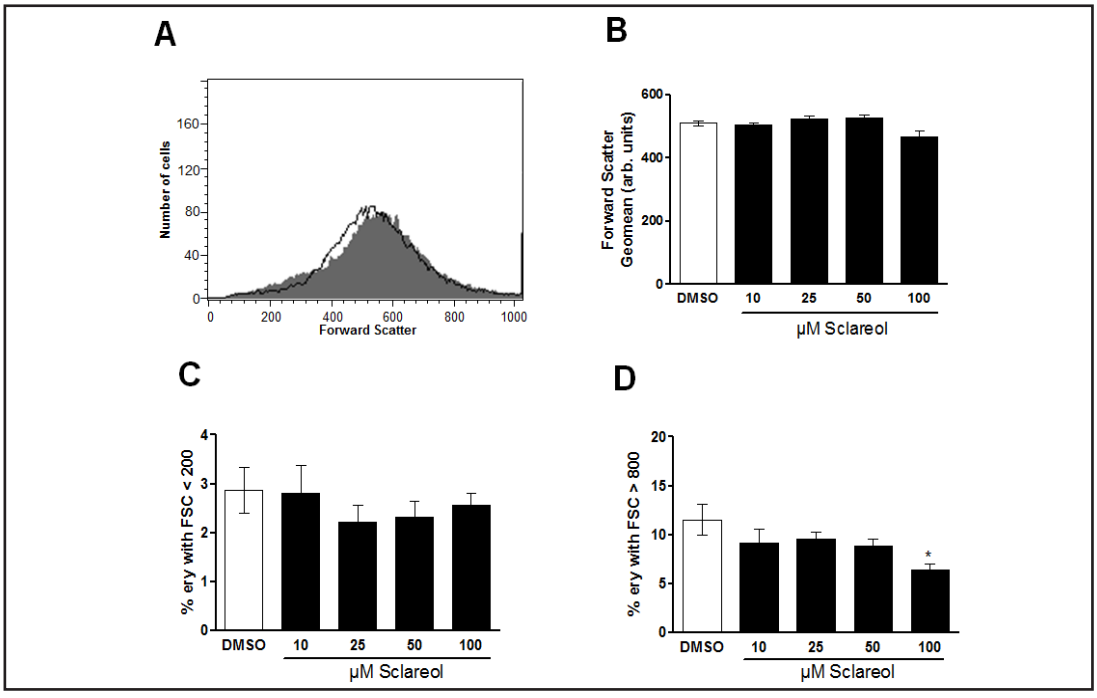

Fig. 2. Effect of Sclareol on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 100 µM Sclareol. B. Arithmetic means ± SEM (n = 10) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Sclareol (10 - 100 µM). C. Arithmetic means ± SEM (n = 10) of the percentage erythrocytes with forward scatter (FSC) <200 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Sclareol (10 – 100 µM). D. Arithmetic means ± SEM (n = 10) of the percentage erythrocytes with forward scatter (FSC) >800 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Sclareol (10 – 100 µM). *(p<0.05) indicates significant difference from the absence of Sclareol (ANOVA).

Fluo3-fluorescence was determined as a measure of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). As illustrated in Fig. 4, a 48 hours exposure to 100 µM Sclareol significantly increased the Fluo3-fluorescence, an observation pointing to increase of $[Ca^{2+}]_i$.

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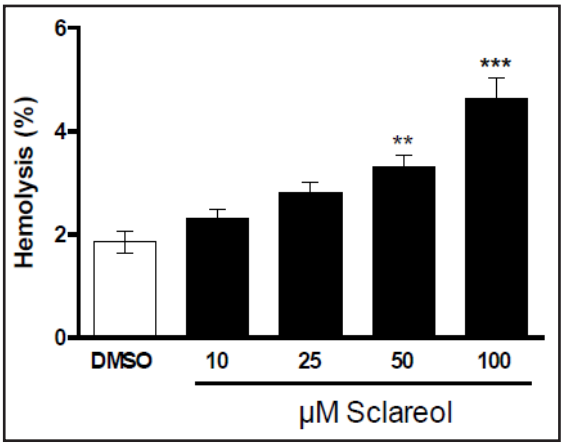

Fig. 3. Effect of Sclareol on hemolysis. Arithmetic means ± SEM (n = 10) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Sclareol (10 - 100 µM). **(p<0.01), ***(p<0.001) indicates significant difference from the absence of Sclareol (ANOVA).

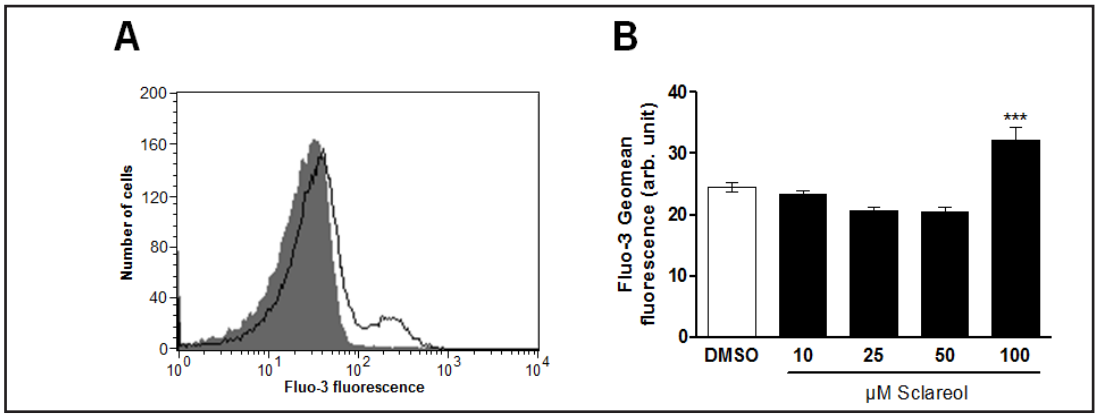

Fig. 4. Effect of Sclareol on cytosolic Ca^{2+} activity. A. Original histogram of Fluo3-fluorescence of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 100 µM Sclareol. B. Arithmetic means ± SEM (n = 10) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Sclareol (10 - 100 µM). ***(p<0.001) indicates significant difference from the absence of Sclareol (ANOVA).

In order to test, whether the Sclareol-induced translocation of phosphatidylserine was sensitive to extracellular Ca^{2+}, erythrocytes were incubated for 48 hours in the absence or presence of 50 or 100 µM Sclareol in the presence or nominal absence of extracellular Ca^{2+}. As shown in Fig. 5, removal of extracellular Ca^{2+} did not significantly blunt the effect of Sclareol on the percentage of annexin-V-binding erythrocytes. Moreover, even in the absence of extracellular Ca^{2+}, Sclareol significantly increased the percentage of annexin-V-binding erythrocytes. Thus, Sclareol-induced cell membrane scrambling was not dependent on 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, the DCF-fluorescence was similar following exposure to 10 µM Sclareol (15.1 ± 0.4 a.u., n = 5), 25 µM Sclareol (13.6 ± 0.6 a.u., n = 5) 50 µM Sclareol (12.8 ± 0.4 a.u., n = 5) and 100 µM Sclareol (17.4 ± 2.5 a.u., n = 5) as in the absence of Sclareol (17.3 ± 2.6 a.u., n = 5). Thus, Sclareol did not appreciably induce 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 similar following exposure to 50 µM Sclareol (10.9 ± 0.2 a.u., n = 5) and 100 µM Sclareol (11.4 ± 0.2 a.u., n = 5) as in the absence of Sclareol (11.2 ± 0.3 a.u., n = 5). Thus, Sclareol did not appreciably induce ceramide abundance.

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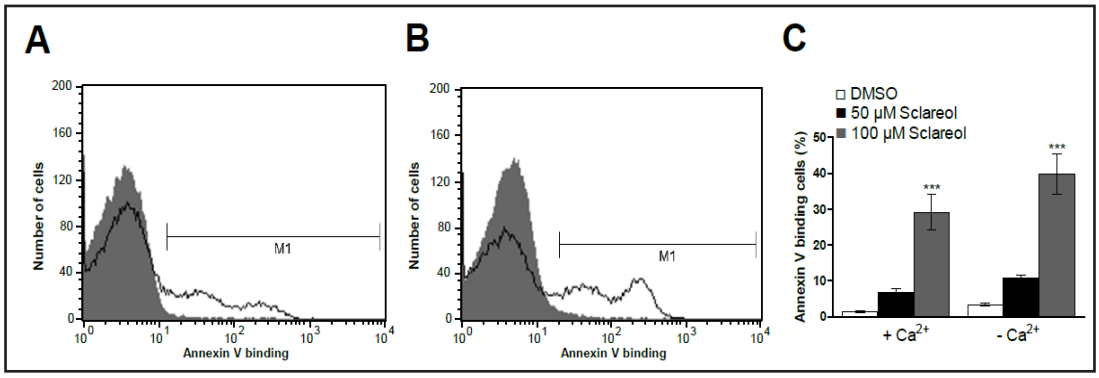

Fig. 5. Ca^{2+} sensitivity of Sclareol -induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) Sclareol (100 µM) in the presence (A) and absence (B) of extracellular 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 50 µM (black bars) or 100 µM (grey bars) Sclareol 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 Sclareol (ANOVA).

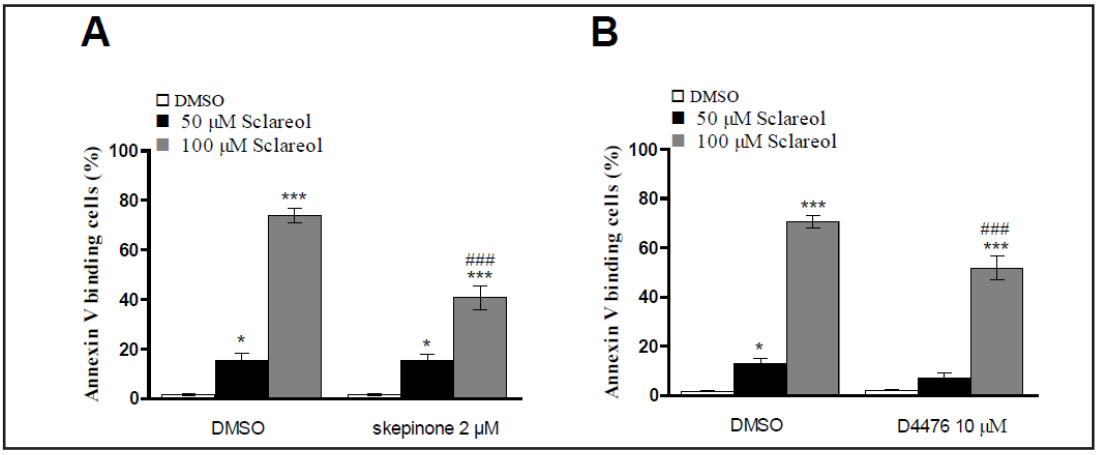

Fig. 6. Effect of p38 kinase inhibitor skepinone and of casein kinase 1α inhibitor D4476 on Sclareol -induced phosphatidylserine exposure. A. Arithmetic means ± SEM (n = 10) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with 50 µM (black bars) or 100 µM (grey bars) Sclareol in the absence (left bars, DMSO) and presence (right bars, skepinone) of p38 kinase inhibitor skepinone (2 µM). B. Arithmetic means ± SEM (n = 15) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with 50 µM (black bars) or 100 µM (grey bars) Sclareol in the absence (left bars, DMSO) and presence (right bars, D4476) of casein kinase 1α inhibitor D4476 (10 µM). *(p<0.05), ***(p<0.001) indicates significant difference from the absence of Sclareol, ###(p<0.001) indicates significant difference from the absence of kinase inhibitors (ANOVA).

To explore, whether the effects of Sclareol involved p38 kinase activity, the influence of Sclareol on annexin-V-binding was tested in the absence or presence of p38 kinase inhibitor skepinone (2 µM). As illustrated in Fig. 6A, the effect of 100 µM Sclareol was significantly blunted in the presence of skepinone. However, even in the presence of skepinone, Sclareol significantly increased the percentage of annexin-V-binding erythrocytes. Thus, Sclareol-induced cell membrane scrambling was apparently in part but not fully due to activation of p38 kinase.

To explore, whether the effects of Sclareol required casein kinase 1α activity, the influence of Sclareol on annexin-V-binding was tested in the absence or presence of casein kinase 1α inhibitor D4476 (10 µM). As illustrated in Fig. 6B, the effect of 100 µM Sclareol

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was significantly blunted in the presence of D4476. However, even in the presence of D4476, Sclareol significantly increased the percentage of annexin-V-binding erythrocytes. Thus, Sclareol-induced cell membrane scrambling was again in part but not fully due to activation of casein kinase 1α.

Discussion

The present observations uncover a novel stimulator of erythrocyte cell membrane scrambling, i.e. the diterpene alcohol Sclareol. Treatment of erythrocytes from healthy individuals with Sclareol results in cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface.

The effect of 100 μM Sclareol on cell membrane scrambling was paralleled by an increase of Fluo3-fluorescence, an observation pointing to increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). However, the effect on cell membrane scrambling was not significantly modified by removal of Ca^{2+} from extracellular space, indicating that the effect did not require entry of extracellular Ca^{2+}. Moreover, the effect of Sclareol on cell membrane scrambling was not paralleled by oxidative stress and not by increased ceramide abundance at the erythrocyte surface. Instead, the effect of Sclareol on cell membrane scrambling was significantly blunted by pharmacological inhibition of p38 kinase and of casein kinase 1α, both kinases known to be involved in the machinery stimulating eryptosis [29]. However, even in the presence of the respective kinase inhibitors, Sclareol significantly increased the percentage of annexin-V-binding erythrocytes, indicating that the stimulation of eryptosis by Sclareol may involve further cellular mechanisms. Those mechanisms could include activation of further kinases, such as protein kinase c or Janus-activated kinase JAK3, as well as inhibition of kinases, such as AMPK, cGMP-dependent protein kinase, and PAK2 kinase [29].

Despite a significant increase of $[Ca^{2+}]_i$ following Sclareol treatment, Sclareol had no significant effect on the average forward scatter. An increase of $[Ca^{2+}]_i$ were expected to activate Ca^{2+} sensitive K^+ channels leading to K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [28]. Possibly, the effect of K^+ channel activation on cell volume was abrogated by impairment of Na^+/K^+ ATPase with respective dissipation of chemical K^+, Na^+ and Cl^- grandients.

Besides its effect on cell membrane scrambling, Sclareol triggered hemolysis with release of hemoglobin, which may *in vivo* pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude nephrons and thus cause renal failure [77]. To the extent that eryptosis precedes hemolysis, those consequences are prevented due to timely removal of the affected erythrocytes from circulating blood.

The loss of phosphatidylserine exposing erythrocytes may, however, lead to anemia as long as it is not compensated by stimulation of erythropoiesis with matching formation of new erythrocytes [29]. Phosphatidylserine exposing erythrocytes further bind to the vascular wall [78], trigger blood clotting and thus predispose to thrombosis [79-81]. Excessive eryptosis could thus impair microcirculation [30, 79, 82-85].

Conclusion

In conclusion, Sclareol triggers cell membrane scrambling, an effect partially dependent on activities of p38 kinase and casein kinase 1α.

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Disclosure Statement

The authors of this manuscript state that they have no conflicts of interest to declare.

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