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Original Paper

Triggering of Suicidal Erythrocyte Death by Zosuquidar

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Key Words
Phosphatidylserine • Calcium • Cell volume • SB203580 • Calphostin • Eryptosis

Abstract
Background: The P-glycoprotein inhibitor zosuquidar (LY335979) is clinically used to augment the effect of cytostatic drugs on suicidal tumor cell death or apoptosis. The present study explored whether the substance is cytotoxic to erythrocytes. Upon injury, erythrocytes may undergo suicidal cell death or eryptosis, which is characterized by cell shrinkage and translocation of cell membrane phosphatidylserine to the erythrocyte surface. Signaling of eryptosis include increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$), oxidative stress and activation of several kinases, such as p38 kinase and protein kinase C. ***Methods:*** Phosphatidylserine abundance at the erythrocyte surface was quantified from binding of FITC-labelled annexin-V, cell volume from forward scatter, $[Ca^{2+}]_i$ from Fluo3-fluorescence, and reactive oxygen species (ROS) from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. ***Results:*** A 48 h treatment of human erythrocytes with zosuquidar significantly increased the percentage of annexin-V-binding cells (2 and 4 µg/ml), significantly decreased forward scatter (4 µg/ml), significantly increased $[Ca^{2+}]_i$ (4 µg/ml), but did not significantly modify ROS. The up-regulation of annexin-V-binding following zosuquidar (4 µg/ml) treatment was significantly blunted by removal of extracellular Ca^{2+}, by presence of p38 kinase inhibitor SB203580 (2 µM) and by presence of protein kinase C inhibitor calphostin (100 nM). ***Conclusions:*** Exposure of erythrocytes to zosuquidar triggers suicidal erythrocyte death with erythrocyte shrinkage and erythrocyte membrane scrambling, an effect involving Ca^{2+} entry and requiring activity of SB203580 and calphostin sensitive kinases.

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Introduction

Zosuquidar (LY335979), a highly specific P-glycoprotein (multi drug resistance) inhibitor [1-11], has been used in the treatment of malignancy [2, 5, 9, 12-26] and interferes with drug efflux at the blood brain barrier [1, 27-29]. Moreover, zosuquidar or its derivatives may be effective on selected bacterial and fungal ABC transporters [30]. The inhibitory effect of zosuquidar on P-glycoprotein impairs the efflux of cytotoxic drugs thus fostering apoptosis of treated cells [30-32].

Similar to apoptosis of nucleated cells erythrocytes may enter suicidal cell death or eryptosis, which is characterized by cell shrinkage [33] and cell membrane scrambling with translocation of phosphatidylserine to the erythrocyte surface [34]. Signaling involved in the stimulation of eryptosis include Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), ceramide [35], oxidative stress [34], caspase activation [34, 36, 37], enhanced activity of casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, or p38 kinase, as well as impaired activity of AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase [34] or sorafenib & sunitinib sensitive kinases [34]. Due to triggering of the respective signaling eryptosis is stimulated by a myriad of xenobiotics [34, 38-73].

The present study explored, whether zosuquidar stimulates eryptosis. To this end, erythrocytes from healthy volunteers were exposed for 48 hours to zosuquidar, and phosphatidylserine abundance at the erythrocyte surface estimated using annexin-V-binding, cell volume from forward scatter, $[Ca^{2+}]_i$ from Fluo3-fluorescence, and abundance of reactive oxygen species (ROS) from 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. The involvement of kinases was tested utilizing p38 kinase inhibitor SB203580 and protein kinase C inhibitor calphostin.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Lithium-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 23°C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* for 48 hours 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, and 1 $CaCl_2$; the pH was adjusted to 7.4 and the temperature kept at 37°C. Where indicated, erythrocytes were exposed to zosuquidar (Sigma Aldrich, Hamburg, Germany, stock solution: 4 mg/ml in water) in the absence or presence of p38 kinase inhibitor SB203580 (Enzo Life Sciences, Farmingdale, USA, stock solution: 20 mM in DMSO) or calphostin (Cayman, Ann Arbor, USA, stock solution:1 mM in DMSO).

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. 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). In some experiments erythrocytes were preincubated in Ca^{2+} free solution. For determination of annexin-V-binding, addition of Ca^{2+} (5 mM $CaCl_2$) was required during the 15 min incubation with FITC-annexin V. Immediately thereafter measurements were done so that the exposure to Ca^{2+} was too short to trigger significant phosphatidylserine translocation.

Intracellular Ca^{2+}

After incubation, a 100 µl cell suspension was washed in Ringer solution and then 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 and washed twice in Ringer solution. 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.

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).

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

The phosphatidylserine abundance at the cell surface was quantified by determination of FITC-labelled annexin-V, which tightly binds to phosphatidylserine. FITC-labelled annexin-V was determined by flow cytometry. As shown in Fig. 1, a 48 hours exposure to zosuquidar increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 2 µg/ml zosuquidar concentration.

Erythrocyte cell volume was estimated from forward scatter determined in flow cytometry. As illustrated in Fig. 2, a 48 hours exposure to zosuquidar was followed by a

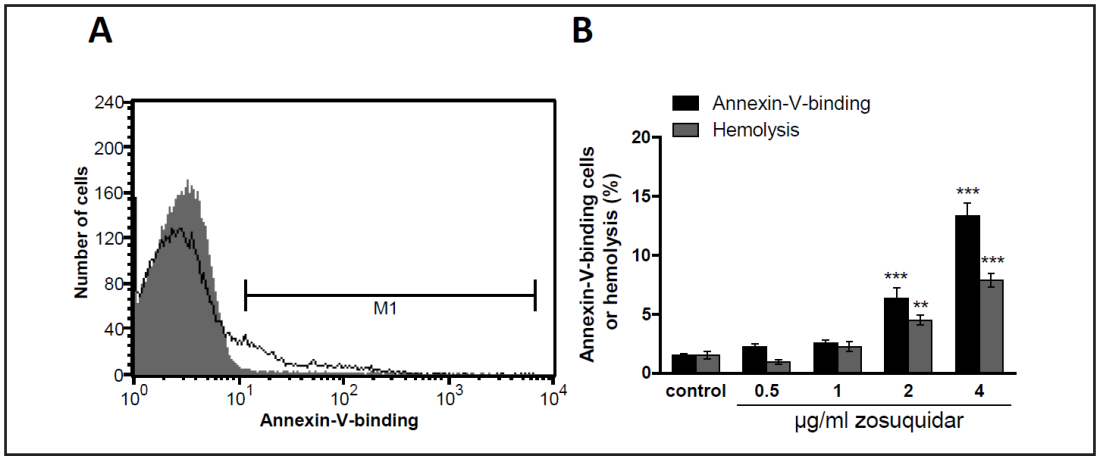

Fig. 1. Effect of zosuquidar on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 4 µg/ml zosuquidar. M1 indicates the annexin-V-fluorescence defining the percentage of annexin-V-binding erythrocytes. B. Arithmetic means ± SEM of erythrocyte annexin-V-binding (n = 7) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of zosuquidar (0.5 - 4 µg/ml). For comparison, the effect of zosuquidar on hemolysis is shown (grey bars). **(p<0.01), ***(*P*<0.001) indicates significant difference from the absence of zosuquidar (ANOVA).

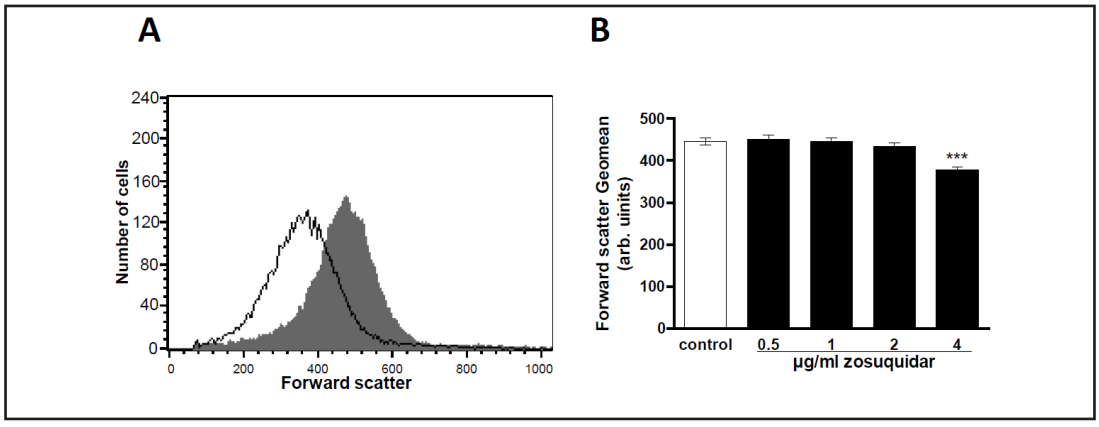

Fig. 2. Effect of zosuquidar on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 4 µg/ml zosuquidar. B. Arithmetic means ± SEM (n = 12) of the erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) zosuquidar (0.5 - 4 µg/ml). ***(P<0.001) indicates significant difference from the absence of zosuquidar (ANOVA).

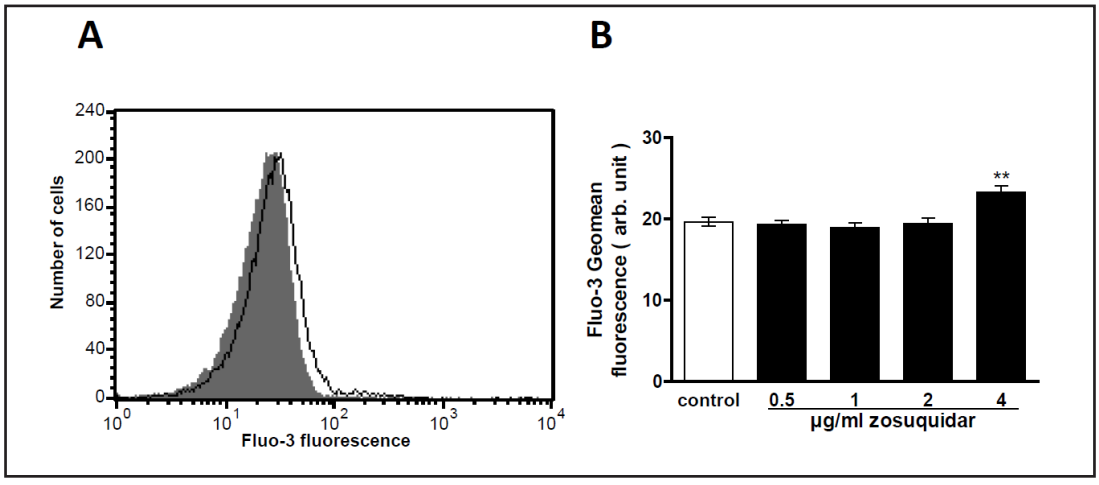

Fig. 3. Effect of zosuquidar on erythrocyte Ca^{2+} activity. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of zosuquidar (4 µg/ml). B. Arithmetic means ± SEM (n = 5) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) zosuquidar (0.5 - 4 µg/ml). **(P<0.01) indicates significant difference from the absence of zosuquidar (ANOVA).

decrease of erythrocyte forward scatter, an effect reaching statistical significance at 4 µg/ml zosuquidar concentration.

Both, cell membrane scrambling and cell shrinkage could be triggered by increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). Fluo3 fluorescence was thus employed to explore whether zosuquidar influences cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). As illustrated in Fig. 3, a 48 hours exposure to zosuquidar increased the Fluo3 fluorescence, an effect requiring 4 µg/ml zosuquidar concentration for statistical significance.

In order to test whether zosuquidar-induced translocation of phosphatidylserine to the cell surface required entry of extracellular Ca^{2+}, erythrocytes were incubated for 48 hours in the absence or presence of 2 or 4 µg/ml zosuquidar, both in the presence or nominal absence of extracellular Ca^{2+}. As illustrated in Fig 4, removal of extracellular Ca^{2+} did not significantly modify the effect of 2 µg/ml zosuquidar, but significantly blunted the effect of 4 µg/ml zosuquidar on annexin-V-binding. Exposure to 2 µg/ml zosuquidar significantly increased the percentage of annexin-V-binding erythrocytes to similarly high levels in the

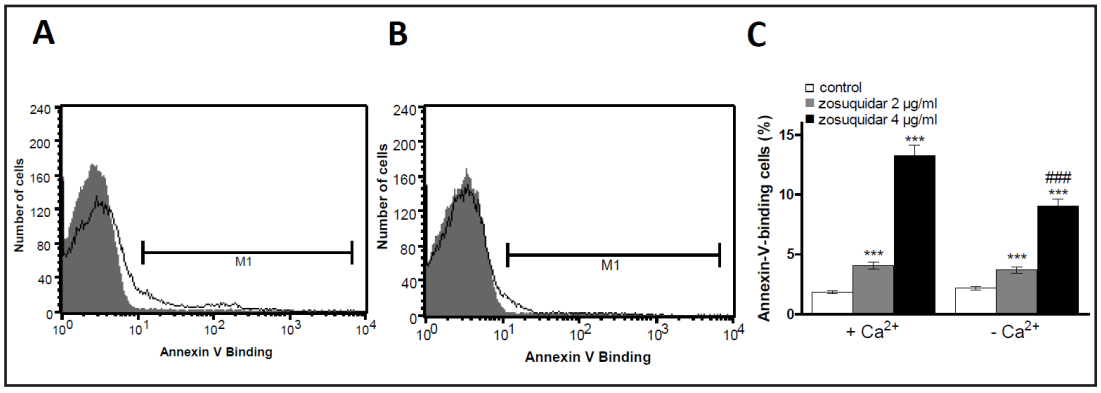

Fig. 4. Ca^{2+} sensitivity of zosuquidar- induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey areas) and with (black lines) presence of zosuquidar (4 µg/ml) in the presence (A) and absence (B) of extracellular Ca^{2+}. C. Arithmetic means ± SEM (n = 12) of annexin-V-binding of erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with 2 µg/ml (grey bars) or 4 µg/ml (black bars) zosuquidar 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 zosuquidar, ###(P<0.001) indicates significant difference from presence of Ca^{2+} (ANOVA).

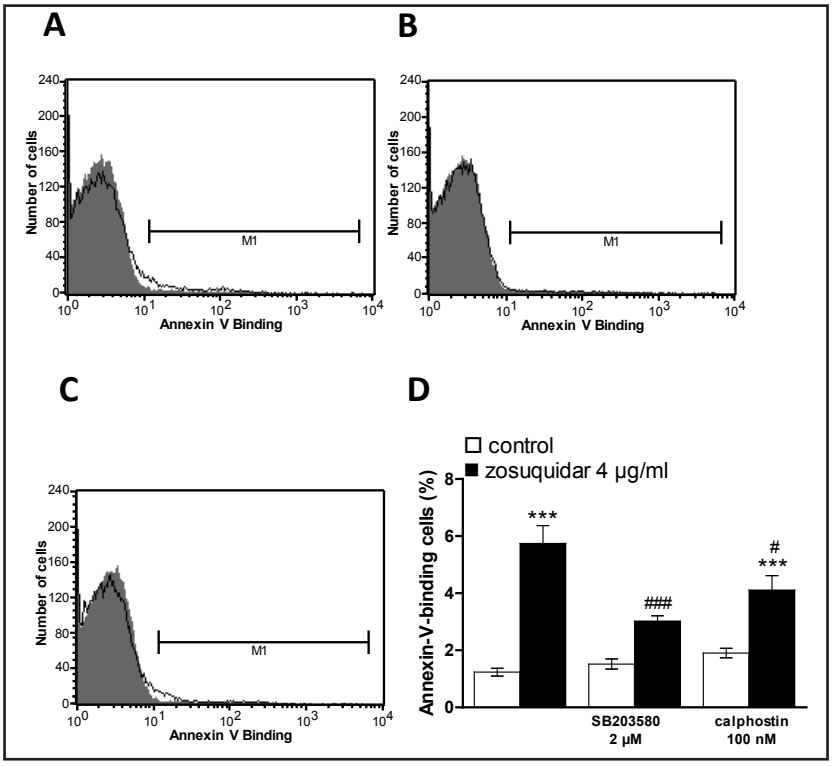

Fig. 5. Effect of zosuquidar on phosphatidylserine exposure in the absence and presence of SB203580 or calphostin. A-C. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey areas) and with (black lines) presence of zosuquidar (4 µg/ml) in the absence of kinase inhibitors (A), in the presence of 2 µM SB203580 (B) or in the presence of 100 nM calphostin (C). D. Arithmetic means ± SEM (n = 13) of annexin-V-binding of erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 4 µg/ml zosuquidar in the absence of kinase inhibitors (left bars) and presence of 2 µM SB203580 (middle bars) or of 100 nM calphostin (right bars). ***(P<0.001) indicates significant difference from the absence of zosuquidar, #(p<0.05), ###(P<0.001) indicates significant difference from the respective value in the absence of kinase inhibitors (ANOVA).

absence and in the presence of extracellular Ca^{2+} and exposure to 4 µg/ml zosuquidar still significantly increased the percentage of annexin-V-binding erythrocytes in the absence of

extracellular Ca^{2+}. Thus, eryptosis was in large part triggered by mechanisms other than entry of extracellular Ca^{2+}.

Eryptosis could be stimulated by oxidative stress. Thus, 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence was utilized to quantify reactive oxygen species (ROS) abundance. As a result, the ROS abundance was similar following a 48 hours incubation in the absence of zosuquidar (17.6 ± 1.9 a.u., n = 5) and in the presence of 0.5 μg/ml (14.7 ± 0.5 a.u., n = 5), 1 μg/ml (15.0 ± 0.4 a.u., n = 5), 2 μg/ml (15.6 ± 0.5 a.u., n = 5) and 4 μg/ml (17.0 ± 0.4 a.u., n = 5) zosuquidar. Thus, zosuquidar did not appreciably enhance oxidative stress.

In order to test, whether the effect of zosuquidar required kinase activation, the effect of zosuquidar on translocation of phosphatidylserine to the cell surface was determined in the absence and presence of p38 protein kinase inhibitor SB203580 (2μM) or protein kinase C inhibitor calphostin (100 nM). As shown in Fig. 5, addition of either SB203580 or calphostin significantly blunted the effect of zosuquidar on annexin-V-binding.

Discussion

The present observations uncover a novel effect of zosuquidar, i.e. the stimulation of eryptosis, the suicidal erythrocyte death characterized by erythrocyte shrinkage and erythrocyte cell membrane scrambling with phosphatidylserine translocation from the cell interior to the erythrocyte surface. The zosuquidar concentration required for stimulation of erythrocyte cell membrane scrambling (2 μg/ml) was in the range of the concentrations determined in mice following treatment with 20 mg/kg zosuquidar [74], but was higher than the concentrations observed in patients [74, 75]. It must be kept in mind that the susceptibility to eryptosis is enhanced in several clinical conditions, such as malignancy [76], hepatic failure [77], diabetes [78, 79], uremia [44, 80], hemolytic uremic syndrome [81], sepsis [82], fever [83], hyperphosphatemia [69], dehydration [61], mycoplasma infection [84], malaria [85], iron deficiency [86], sickle cell anemia [87], thalassemia [87], glucose-6-phosphate dehydrogenase deficiency [87], and Wilson´s disease [34, 88]. In those conditions, presumably lower concentrations of zosuquidar are required to trigger eryptosis.

Signaling involved in zosuquidar induced eryptosis includes increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), which may contribute to stimulation of cell membrane scrambling by activating an illdefined Ca^{2+} sensitive scramblase and of cell shrinkage by activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [33]. Accordingly, removal of extracellular Ca^{2+} slightly but significantly blunted the stimulation of annexin-V-binding following treatment with 4 μg/ml zosuquidar. However, removal of extracellular Ca^{2+} did not appreciably influence the effect of 2 μg/ml zosuquidar and 4 μg/ml zosuquidar still significantly enhanced the phosphatidylserine abundance at the cell surface in the absence of extracellular Ca^{2+}. Thus, the effect of zosuquidar on Ca^{2+} entry contributed only little to the stimulation of phosphatidylserine translocation.

Moreover, zosuquidar failed to trigger oxidative stress, a known stimulator of eryptosis [34]. Instead, the effect of zosuquidar on cell membrane scrambling apparently involved activation of kinases. The effect of zosuquidar on cell membrane scrambling was significantly blunted by the p38 kinase inhibitor SB203580 and the protein kinase C inhibitor calphostin.

The stimulation of eryptosis by zosuquidar may lead to anemia, as phosphatidylserine exposing erythrocytes are engulfed by macrophages and thus rapidly cleared from circulating blood [34]. Erythrocytes exposing phosphatidylserine at their surface may further adhere to endothelial cells of the vascular wall [89], stimulate blood clotting and induce thrombosis [90-92]. The stimulation of erythrocyte cell membrane scrambling may thus interfere with microcirculation [35, 90, 93-96].

In conclusion, exposure of human erythrocytes to zosuquidar is followed by stimulation of erythrocyte cell membrane scrambling and cell shrinkage, both hallmarks of eryptosis, the suicidal erythrocyte death. Signaling involved in the effect of zosuquidar on cell membrane scrambling includes Ca^{2+} entry, p38 kinase and protein kinase C.

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Disclosure Statement

The authors declare no conflict of interest.

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