

Original Paper

Triggering of Suicidal Erythrocyte Death by Pazopanib

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Phosphatidylserine • Cell volume • Eryptosis • Ionomycin • Calcium

Abstract

Background/Aims: The multi-targeted kinase inhibitor pazopanib, a drug employed for the treatment of a wide variety of malignancies, has previously been shown to trigger apoptosis. Similar to apoptosis of nucleated cells, erythrocytes may enter suicidal death or eryptosis, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Mechanisms involved in the triggering of eryptosis include Ca^{2+} entry, oxidative stress and ceramide. The present study explored, whether pazopanib induces eryptosis and, if so, whether it is effective by Ca^{2+} entry, oxidative stress and/or ceramide. **Methods:** Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, reactive oxygen species (ROS) formation from DCF dependent fluorescence, and ceramide abundance utilizing specific antibodies. **Results:** A 48 hours exposure of human erythrocytes to pazopanib significantly increased the percentage of annexin-V-binding ($\geq 25 \mu\text{g/ml}$) and of shrunken erythrocytes ($\geq 50 \mu\text{g/ml}$). Pazopanib treatment further resulted in significant hemolysis ($\geq 25 \mu\text{g/ml}$). The effect of pazopanib on annexin-V-binding was significantly blunted but not abolished by removal of extracellular Ca^{2+} . Pazopanib significantly increased DCF fluorescence ($50 \mu\text{g/ml}$) and ceramide abundance ($50 \mu\text{g/ml}$). **Conclusions:** Pazopanib triggers eryptosis, an effect involving Ca^{2+} entry, oxidative stress and ceramide.

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Pazopanib, a multi-targeted inhibitor of tyrosine kinase [1-13], vascular endothelial growth factor receptor [4, 8, 14-17], and angiogenesis [2, 11, 18-21], is used for treatment of diverse malignancies [8], including renal cell carcinoma [1, 3-5, 7, 9, 13, 20, 22-38], soft

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tissue sarcoma [2, 39-44], recurrent multiple CNS hemangioblastomas [45], epithelial ovarian cancer [46], gastroenteropancreatic neuroendocrine tumours [47], malignant glioma [48], urothelial cancer [49], breast cancer [50], non small cell lung carcinoma [51] and medulloblastoma [52]. Untoward side effects of pazopanib include alopecia [53], hypertension [10, 21, 33, 49], fatigue [21, 49, 54], gastrointestinal events [10, 21, 33, 49, 55, 56], liver toxicity [10, 33, 57] and hand-foot-skin reaction [6].

Pazopanib has been shown to stimulate apoptosis [51, 58-66], but may inhibit necroptosis [67]. Mechanisms involved in the stimulation of apoptosis include downregulation of the antiapoptotic proteins XIAP and MCL1 [63], as well as of HIF1 α and ABCG2 genes [57], mammalian target of rapamycin [68], and poly(ADP-ribose) polymerase cleavage [63]. Mechanisms involved in the inhibition of necroptosis include receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and 3 (RIPK3) as well as transforming growth factor- β -activated kinase 1 (TAK1) [67].

Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis [69], the suicidal death of erythrocytes characterized by cell shrinkage [70] and cell membrane scrambling apparent from phosphatidylserine translocation to the cell surface [69]. Eryptosis is triggered by Ca²⁺ entry [69], ceramide [71], oxidative stress [69], energy depletion [69], activated caspases [69, 72, 73], and activation of some kinases, such CK1 α , JAK3, PKC, p38 kinase and PAK2 kinase [69]. Eryptosis is further stimulated by genetic or pharmacological knockout of AMPK, cGMP-dependent protein kinase, and sorafenib/sunitinib sensitive kinases [69]. Eryptosis is stimulated by a variety of xenobiotics [69, 74-114].

The present study explored, whether pazopanib stimulates eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to pazopanib and phosphatidylserine surface abundance, cell volume, formation of reactive oxygen species (ROS), and ceramide abundance quantified utilising 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 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 CaCl₂, at 37°C for 48 hours. Where indicated, erythrocytes were exposed for 48 hours to pazopanib (MedChem Express, Princeton, USA). In order to estimate the impact of pazopanib on eryptosis due to high [Ca²⁺]_i, erythrocytes were exposed for 1 hour to a combination of pazopanib and the Ca²⁺ ionophore ionomycin (Merck Millipore, Darmstadt, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 100 μ l cell suspension was washed in Ringer solution containing 5 mM CaCl₂ 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 pazopanib 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".

Hemolysis

For the determination of hemolysis, the samples were centrifuged (10 min at 2000 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.

Fluo3-fluorescence

After incubation, erythrocytes were washed in Ringer solution and loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 μ M Fluo-3/AM. The cells were incubated at 37°C for 30 min. Ca²⁺-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 μ l suspension of erythrocytes was washed in Ringer solution and stained with DCF diacetate (Sigma, Schnelldorf, Germany) in Ringer solution containing DCF diacetate at a final concentration of 10 μ M. Erythrocytes were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCF-loaded erythrocytes were resuspended in 200 μ l 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 μ g/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.

Statistics

Data are expressed as arithmetic means \pm 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 the tyrosine kinase inhibitor pazopanib triggers eryptosis, the suicidal erythrocyte death characterized by phosphatidylserine translocation to the cell surface and cell shrinkage.

Phosphatidylserine at the erythrocyte surface was estimated from annexin-V-binding which was determined by flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with pazopanib (10 – 50 μ g/ml). As illustrated in Fig. 1, a 48 hours exposure to pazopanib increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 25 μ g/ml pazopanib.

Forward scatter determined by flow cytometry was taken as a measure of erythrocyte volume. Measurements were done in erythrocytes incubated for 48 hours in Ringer solution without or with pazopanib (10 – 50 μ g/ml). The average erythrocyte forward scatter was similar without pazopanib treatment (497 ± 5.6 , *n* = 16) and following treatment with 10 μ g/ml (511 ± 4.8 , *n* = 16), 25 μ g/ml (505 ± 5.0 , *n* = 16), and 50 μ g/ml (476 ± 7.0 , *n* = 16) pazopanib. Moreover, the percentage of cells with forward scatter > 800 was similar without pazopanib treatment (96.4 ± 0.6 , *n* = 16) and following treatment with 10 μ g/ml (95.5 ± 0.5 ,

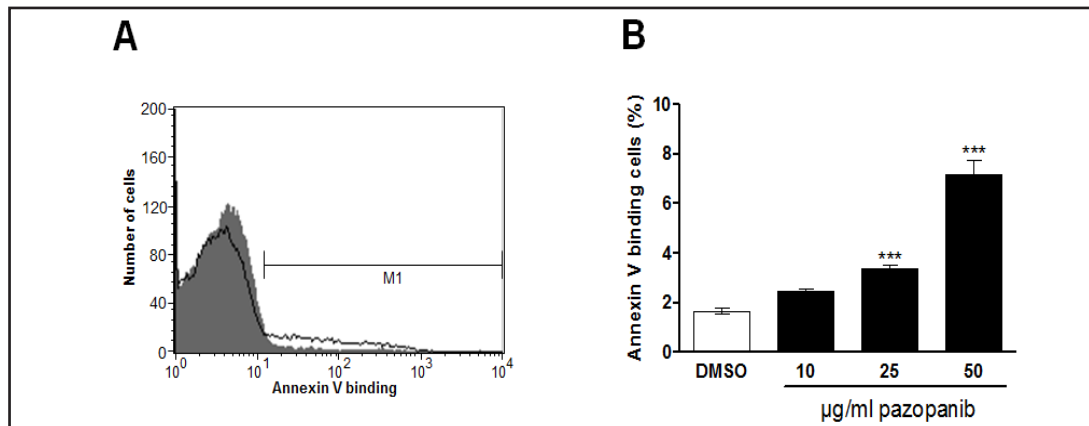


Fig. 1. Effect of pazopanib on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 50 µg/ml pazopanib. B. Arithmetic means ± SEM (n = 16) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) pazopanib (10 - 50 µg/ml). *** (p<0.001) indicates significant difference from the absence of pazopanib (ANOVA).

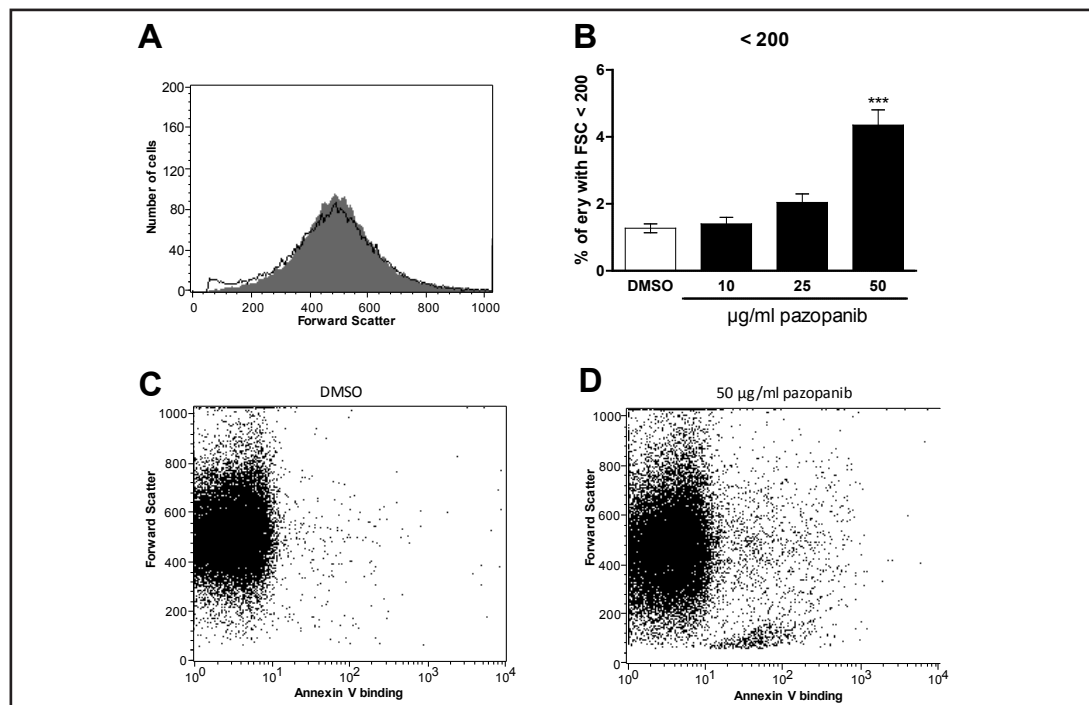


Fig. 2. Effect of pazopanib on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 50 µg/ml pazopanib. B. Arithmetic means ± SEM (n = 16) of the percentage erythrocytes with forward scatter (FSC) < 200 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) pazopanib (10 - 50 µg/ml). C, D. Original dot plots of forward scatter vs annexin V abundance without (C) and with (D) prior treatment with 50 µg/ml pazopanib. *** (p<0.001) indicates significant difference from the absence of pazopanib (ANOVA).

n = 16), 25 µg/ml (95.1 ± 0.6, n = 16), and 50 µg/ml (94.9 ± 0.7, n = 16) pazopanib. However, pazopanib increased the percentage of severely shrunken erythrocytes (Fig. 2A,B), an effect reaching statistical significance at 50 µg/ml pazopanib concentration. Dot blots of annexin-V-binding versus forward scatter reveal that shrunken cells coincide with annexin-V-binding cells (Fig. 2C,D).

Fig. 3. Effect of pazopanib on hemolysis. Arithmetic means \pm SEM (n = 8) of the percentage hemolytic erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) pazopanib (10 - 50 μ g/ml). ***($p < 0.001$) indicates significant difference from the absence of pazopanib (ANOVA).

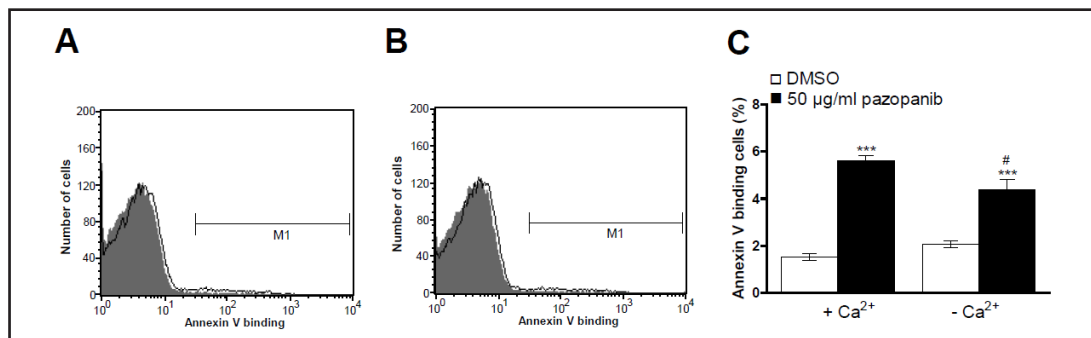
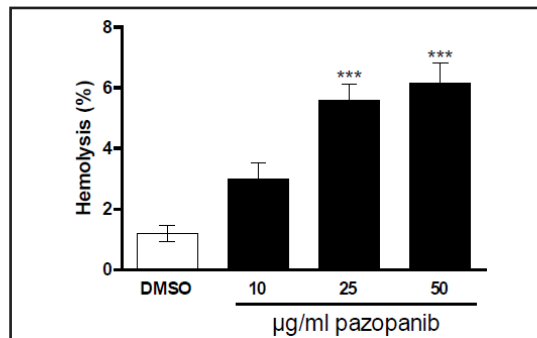


Fig. 4. Ca²⁺ sensitivity of pazopanib -induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) pazopanib (50 μ g/ml) in the presence (A) and absence (B) of extracellular Ca²⁺. C. Arithmetic means \pm SEM (n = 8) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) pazopanib (50 μ g/ml) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. ***($p < 0.001$) indicates significant difference from the absence of pazopanib, #($p < 0.05$) indicates significant difference from the presence of Ca²⁺ (ANOVA).

In order to estimate the impact of pazopanib on hemolysis, the percentage of haemolytic erythrocytes was estimated from the hemoglobin concentration in the supernatant. As illustrated in Fig. 3, pazopanib increased the percentage of haemolytic erythrocytes, an effect reaching statistical significance at 25 μ g/ml pazopanib.

Fluo3-fluorescence was taken as a measure of cytosolic Ca²⁺ activity ([Ca²⁺]_i). As a result, following a 48 hours incubation the Fluo3-fluorescence was lower in the presence of 50 μ g/ml pazopanib (17.8 \pm 2.7 a.u., n = 12) than in the absence of pazopanib (20.1 \pm 3.6 a.u., n = 12). Additional experiments were performed in order to elucidate whether pazopanib affects Fluo3-fluorescence of erythrocytes treated with the Ca²⁺ ionophore ionomycin (1 μ M) and thus containing saturating [Ca²⁺]_i. As a result, 50 μ g/ml pazopanib treatment decreased the Fluo3-fluorescence from 23.1 \pm 1.4 a.u. (n = 5) to 16.5 \pm 0.6 a.u. (n = 5) in the absence of ionomycin and from 46.1 \pm 3.5 a.u. (n = 5) to 33.3 \pm 1.7 a.u. (n = 5) in the presence of ionomycin. This observation suggests that pazopanib interferes with Fluo3-fluorescence by mechanisms other than decreasing [Ca²⁺]_i, such as quenching of the Fluo3-fluorescence or leakage of dye thus reducing Fluo3-fluorescence.

A next series of experiments explored whether the pazopanib-induced translocation of phosphatidylserine to the erythrocyte surface required entry of extracellular Ca²⁺. To this end, erythrocytes were incubated for 48 hours in the absence or presence of 50 μ g/ml pazopanib in the presence or nominal absence of extracellular Ca²⁺. As illustrated in Fig. 4, removal of extracellular Ca²⁺ significantly blunted the effect of pazopanib on annexin-V-binding. However, even in the absence of extracellular Ca²⁺, pazopanib significantly increased the percentage of annexin-V-binding erythrocytes. Thus, pazopanib-induced cell membrane scrambling was in part but not fully dependent on entry of extracellular Ca²⁺.

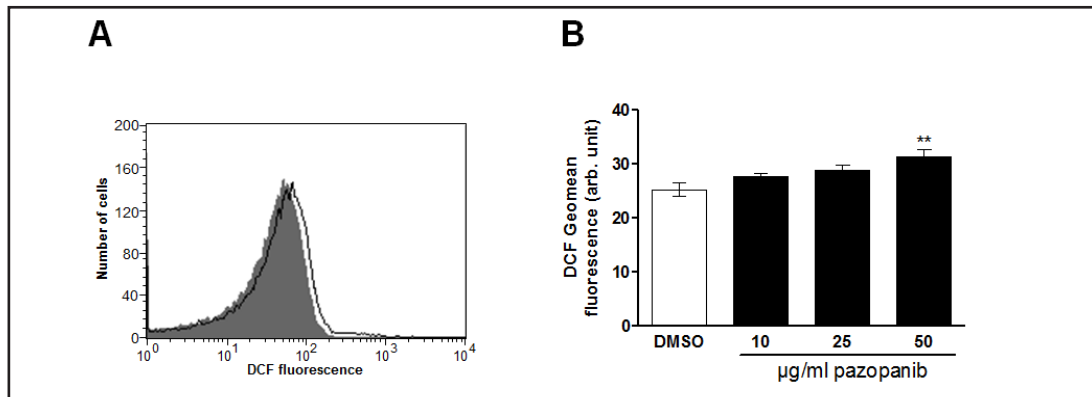


Fig. 5. Effect of pazopanib on ROS formation. A. Original histogram of DCF fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of pazopanib (50 µg/ml). B. Arithmetic means ± SEM (n = 12) of the DCF fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) pazopanib (10 - 50 µg/ml). **($p < 0.01$) indicates significant difference from the absence of pazopanib (ANOVA).

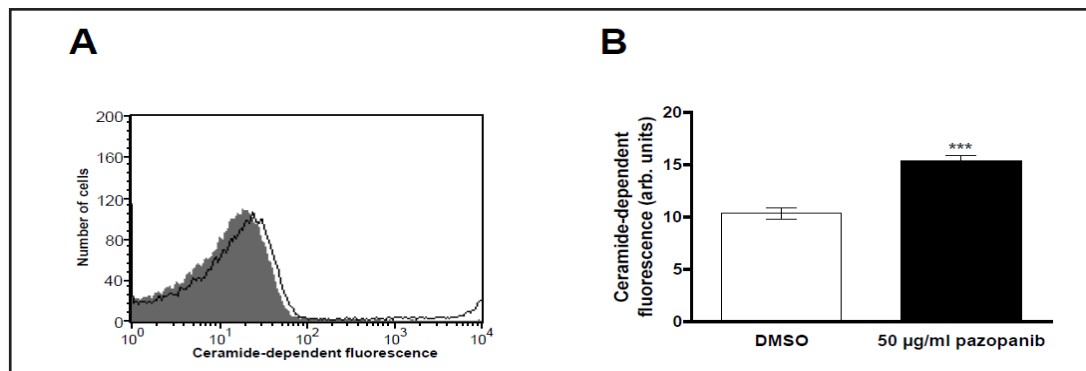


Fig. 6. Effect of pazopanib on ceramide abundance at the erythrocyte surface. A. Original histogram of ceramide abundance in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 50 µg/ml pazopanib. B. Arithmetic means ± SEM (n = 8) of the ceramide abundance (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) presence of 50 µg/ml pazopanib. ***($p < 0.001$) indicates significant difference from the absence of pazopanib (ANOVA).

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) were thus quantified utilizing 2',7'-dichlorodihydrofluorescein (DCF) diacetate. As illustrated in Fig. 5, pazopanib increased the DCF fluorescence in erythrocytes, an effect reaching statistical significance at 50 µg/ml pazopanib.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As illustrated in Fig. 6, 50 µg/ml pazopanib significantly increased the ceramide abundance at the erythrocyte surface.

Discussion

The present observations reveal a novel effect of pazopanib, i.e. the stimulation of suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to pazopanib is followed by increase of the percentage shrunken and phosphatidylserine exposing erythrocytes. The pazopanib concentrations required for triggering of eryptosis are in the range of the plasma concentrations under pazopanib treatment [115, 116].

The effect of pazopanib on cell membrane scrambling was blunted by removal of extracellular Ca^{2+} , an observation suggestive for dependence on Ca^{2+} entry from the extracellular space. Surprisingly, Fluo3-fluorescence decreased following pazopanib treatment. As a control, erythrocytes pretreated without or with pazopanib were exposed to the Ca^{2+} ionophore ionomycin in order to enhance cytosolic Ca^{2+} concentrations to values fully saturating the fluorescent dye. As a result, pazopanib treatment still decreased Fluo3-fluorescence indicating that the substance either interacts with Fluo3-fluorescence or with dye loading. Thus, Fluo3-fluorescence did not yield reliable data on cytosolic Ca^{2+} activity. In any case, the effect of pazopanib on cell membrane scrambling in erythrocytes was partially, but not completely dependent on extracellular Ca^{2+} . Thus additional mechanisms must be involved in the stimulation of cell membrane scrambling by pazopanib. According to the present observations, pazopanib induces oxidative stress and ceramide, both well-known triggers of eryptosis [69].

Pazopanib did not significantly modify the average forward scatter but was followed by severe shrinkage of a small subpopulation of erythrocytes. The shrinkage could have been due to activation of K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [69].

Besides triggering eryptosis, pazopanib stimulates hemolysis. *In vivo*, eryptosis serves to clear defective erythrocytes from circulating blood prior to hemolysis [69]. Hemolysis is followed by release of hemoglobin which may pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude nephrons and thus trigger renal failure [117].

Enhanced eryptosis may lead to anemia, as phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood [69]. Moreover, phosphatidylserine exposing erythrocytes adhere to the vascular wall [118], trigger blood clotting and thus induce thrombosis [119-121]. Eryptotic erythrocytes thus impair microcirculation [71, 119, 122-125].

The effect of pazopanib treatment on eryptosis may be particularly relevant in clinical conditions with enhanced eryptosis, such as dehydration [126], hyperphosphatemia [127], chronic kidney disease (CKD) [128-131], hemolytic-uremic syndrome [132], diabetes [133], hepatic failure [134], malignancy [69], sepsis [135], sickle-cell disease [69], beta-thalassemia [69], Hb-C and G6PD-deficiency [69], as well as Wilsons disease [136].

In conclusion, pazopanib triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect apparently in part dependent on Ca^{2+} entry, oxidative stress and ceramide.

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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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